

SODIUM AND GLUCOSE TRANSPORT ACROSS
THE IN VITRO PERFUSED MIDGUT OF THE
BLUE CRAB, CALLINECTES SAPIDUS RATHBUN

by

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TO MY FAMILY

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ABSTRACT

The principal objectives of the present study are: (1) To investigate sodium transport across the midgut as well as the hindgut of Callinectes sapidus and the functioning of these two regions of the gut in osmotic and ionic regulation of the animal, and (2) to elucidate the mechanism of glucose absorption across the midgut of C. sapidus and to assess its role in nutrient absorption. The transport processes across the gut tissues were studied with an in vitro perfusion technique.

I demonstrated a net mucosal to serosal sodium transport across the midgut of C. sapidus. This net flux was within the same order of magnitude as the fluxes of isolated gut tissues of many other animal species. The flux displayed saturation kinetics and was ouabain-sensitive. The flux did not vary with environmental salinity, thus providing no evidence for an active regulatory role of the midgut in sodium balance. A reduction of passive permeability with salinity, however, was suggested. There was no measurable net sodium transport across the hindgut of the animal.

The midgut of C. *sapidus* was also capable of net transmural glucose absorption. The net flux was considerably less than the fluxes reported in mammalian intestine. The unidirectional mucosal to serosal glucose flux was depressed by metabolic inhibitors, the absence of mucosal Na⁺, and the presence of mucosal phlorizin or serosal ouabain. The flux was also decreased by the presence of mucosal D-galactose, but not D-fructose or L-glucose. The net glucose flux followed Michaelis-Menten kinetics.

The mucosal glucose uptake by the midgut was composed of two processes: a sodium-dependent, saturable component, and a sodium-independent, non-saturable counterpart. The kinetic characteristics of the uptake suggest that the midgut does not play a major role in total nutrient absorption. Nutrients taken up by the midgut may be largely utilized to provide energy for other physiological functions served by this tissue.

Thin layer chromatographic analyses showed that whereas most glucose appeared as phosphorylated forms upon entering the midgut, free glucose was translocated across the tissue. These results suggest that the mucosal and serosal membrane transport processes are coupled to phosphorylation-dephosphorylation mechanisms, respectively. An alternative hypothesis is the presence of a high-affinity glucose carrier in the serosal border of the midgut.

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CHAPTER I

GENERAL INTRODUCTION

The subject of gastrointestinal physiology of Crustacea has been a field of study for comparative physiologists for more than fifty years. Major emphasis has been placed on the digestive process, especially the identification of digestive enzymes (see Vonk, 1960; van Weel, 1970; Dall & Moriarty, 1983 for reviews). Relatively little is known about the absorptive process by the gut, the subject of my dissertation. In the first part of this chapter, I will review studies on water and solute transport in crustacean gut, paying special attention to information pertinent to the present study. Some of the recent studies in this field have been reviewed by Ahearn (1982). In the second part, the objectives and significance of my study will be discussed.

To facilitate discussion on transport physiology of the crustacean gut, its structure is first briefly described. The digestive tract of Crustacea is composed of three main regions: foregut, midgut, and hindgut. The division is based upon the embryonic origin of the regions. The foregut and the hindgut are derived from the ectodermal epithelium and hence are lined with cuticle. The midgut and its appendages are endodermal in origin and thus lack a chitinous lining. The foregut is short and is commonly enlarged in Malacostraca to form a stomach (proventriculus), which may be divided into anterior and posterior chambers (cardiac and pyloric stomachs), provided with elaborate grinding and filtering mechanisms. The midgut is tubular, bearing one or more outpocketings known as midgut ceca and/or diverticula. Most malacostracans possess a pair of well-developed granular organs known as the hepatopancreas (midgut or digestive gland),

which is generally believed to be the principal organ for secretion of digestive enzymes, and absorption and storage of nutrients (see van Weel, 1974; Gibson & Barker, 1979 for reviews). The hindgut is usually a straight tube of variable length depending on species. The foregoing description is based on Clarke (1979) and McLaughlin (1983).

Histologically, the crustacean gut generally consists of a luminal epithelium surrounded by connective tissues and muscle fibers. Studies with electron microscopy revealed that the gut epithelium usually consists of a single layer of cells joined by junctional complexes at the apical margin, and separated by lateral intercellular spaces (Komuro & Yamamoto, 1968; Georgi, 1969; Koulis, 1971; Talbot, Clark & Lawrence, 1972; Hootman & Conte, 1974; Holdich & Mayes, 1975; Quaglia, Sabelli & Villani, 1976; Mykles, 1977, 1979; Johnson, 1980; see Gibson & Barker, 1979 for a review on the hepatopancreas). Commonly observed features include microvillous apical borders, and elaborate infoldings of the lateral and basal membranes that are closely associated with numerous mitochondria. Thus, the ultrastructure of the crustacean gut resembles that of other epithelia capable of vectorial transport of solutes and water (see Berridge & Oschman, 1972).

Ion and Water Transport in Crustacean Gut

For many years, there have been suggestions in the literature that the crustacean gut is an organ of salt and water balance (see Mantel & Farmer, 1983 for a review). Most of these assertions have been based on histological observations, drinking rate determinations, and analyses of osmotic and ionic concentrations of the gut fluid of various species. It has long been known that many crustaceans drink their ambient medium (Fox, 1952; Dall & Smith, 1977). Croghan (1958) found that the homeosmotic brine shrimp, Artemia salina, ingests the medium, and that its gut fluid maintains a higher osmotic pressure but a lower NaCl concentration than the hemolymph. He accounted for this by postulating NaCl and water absorption through the gut. Geddes (1975) reported a similar role of the gut in osmoregulation of Parartemia zietziana. However, in contrast to A. salina, the gut fluid is isosmotic to the hemolymph.

Lindquist & Fitzgerald (1976) showed that in the terrestrial isopod Porcellio scaber, the gut fluid acts as a buffer between the hemolymph and the external environment, such that water is withdrawn from the gut lumen on desiccation. Farmer (1980) found that the copepod Acartia tonsa regulates a lower hemolymph Na^+ level in hypersaline media. Under these conditions, the copepod increases its drinking rate, which is amiloride-sensitive, indicating that water loss in hyporegulation is replaced by Na^+ -dependent fluid absorption by the gut.

Fiddler crabs of the genus Uca have a wide range of salinity tolerance, and regulate hyperosmotically in brackish water and

hyposmotically in higher salinities (Jones, 1941). Based on analysis of stomach fluid, Green et al. (1958) suggested that both Na^+ and water are probably absorbed by the foregut of hyposmotic Uca. Hannan & Evans (1973) demonstrated that Uca ingests the external medium. Baldwin & Kirschner (1976a, b) showed that drinking rate of Uca adapted to 175% seawater is higher than that of animals adapted to 10% seawater. Thus, this study also indicated that water absorption takes place in the gut of hyposmotic Uca.

Dall (1967) found that the hyporegulatory penaeid shrimp, Metapenaeus bennettiae, increases its drinking rate in water of higher salinity. Chemical analysis and ^{22}Na , ^{36}Cl uptake in the tissues indicated that the anterior diverticula of the midgut are salt secretory. Experiments on Ca^{45} uptake also suggested that the tubular midgut is an active site of calcium exchange (Dall, 1965). Further, he found that when the crab, Scylla serrata, was given a hyperosmotic salt load, its gut was distended with a fluid of a higher Cl^- concentration than that of the gut fluid of an individual without the salt load, indicating Cl^- secretion through the gut (Dall, 1967). This is in contrast with the western rock lobster, Panulirus longipes, where Cl^- concentration is reduced in gut fluid following a salt load (Dall, 1974). Excess Ca^{2+} may be secreted by the foregut of this species. Similar salt-loading experiments indicated that excess ions are probably secreted by the gut of the American lobster, Homarus americanus (Dall, 1970). Using a different approach, Malley (1977b) replaced the stomach fluid of the spiny lobster, Panulirus argus, by seawater containing polyethylene glycol as a non-absorbable volume marker and subsequently analysed the changes of ion concentrations with time. These experiments

provided evidence that the foregut absorbs water as well as salts. The rate of this route of water uptake, as measured by the drinking rate, however, accounts for only a minor portion of the total water intake required to balance water loss through urine production.

The work discussed so far indicates that the gut may be capable of polarized ion and water transport. Yet the techniques used in these studies have limitations in the investigation of gut transport processes. As both water and ion movements may be involved in gut transport, chemical analysis of gut fluid can hardly distinguish between absorptive and secretory phenomena. For instance, a higher osmotic pressure in the gut fluid than that in the hemolymph may be the result of either water absorption or salt secretion. The results of salt-loading experiments are especially difficult to interpret as the animals are subjected to non-physiological impact. Appearance of salts in the gut after these experiments may be due to simple diffusion. Moreover, when drinking is always taken as evidence for water absorption by the gut, the gut's ability for fluid uptake cannot be ascertained. For these reasons, the study of ion and water transport properties using isolated gut tissues has become a profitable approach.

Using a ligated sac technique, Yonge (1924) demonstrated fluid absorption across the midgut of the Norwegian lobster, Nephrops norvegicus. This capability is not exhibited by the hindgut. Adapting a similar approach, Malley (1977b) showed limited water and ion movements in the isolated hindgut of P. argus. Based on this observation and her experiments on the foregut (see above), she concluded that the gut is of minimal importance in osmotic and ionic balance for this animal.

Mantel (1968), using an in vitro sheet preparation, showed that the foregut of the terrestrial crab, Gecarcinus lateralis, is permeable to Na^+ , Cl^- and water. The foregut of eyestalkless crabs after ecdysis, however, has a low water permeability, which increases in response to extracts of the thoracic ganglionic mass. She suggested that the permeability of the foregut and probably the direction of net water and ion movements are under neuroendocrine control. Kamemoto (1976) also reported that neuroendocrine factors influence water movement in the isolated hindgut of the crayfish, Procambarus clarkii.

A problem that the in vitro technique has recently elucidated is the role of the midgut ceca in salt and water balance. The functioning of these organs in osmotic and ionic regulation has been speculated by many authors (Smith, 1978; Mykles, 1977, 1979; Johnson, 1980; see also Dall, 1967 above). Heeg & Cannone (1966) proposed that the midgut ceca of two grapsid crabs, Cyclograpsus punctatus and Plagusia chabrus, eliminate water in a low salinity environment. Their evidence, based on morphological observations, is preliminary (Smith, 1978). Recently, Holliday et al. (1980) demonstrated a roughly isosmotic fluid secretion, in vitro and in vivo, in both the anterior and posterior midgut ceca in the Dungeness crab, Cancer magister. The rate of fluid production was low and did not change when crabs were exposed to hyposmotic medium. Thus, they concluded that the midgut ceca contribute little to osmoregulation, and speculated that the ceca may function in water absorption at molt.

During ecdysis in Crustacea, there is a rapid increase in body volume as a result of fluid absorption. The site of this water uptake is, however, obscure. Robertson (1960) found that the shore crab, Carcinus maenas, ingests water consistently at molt but not at intermolt. He suggested that the gut is the major site of fluid absorption at ecdysis. Dall & Smith (1978) showed that the drinking rate of the western rock lobster, Panulirus longipes, increases immediately after molting. Since no evidence of fluid absorption was found in other regions of the gut, they hypothesized that the hepatopancreas is the site of water uptake. The increase in drinking rate, however, cannot account for the total water uptake by the animal. Therefore, they suggested that water probably also enters through other body surfaces. Using an in vitro perfusion technique, Mykles & Ahearn (1978) found that in the freshwater shrimp, Macrobrachium rosenbergii, the rate of net water flux across the midgut is highest in animals approaching ecdysis and decreases after molt. Mykles (1980) found a similar relationship between net water absorption across the midgut and the stage of the molt cycle in the American lobster, Homarus americanus. The increase in this water uptake at ecdysis corresponds to an elevation of drinking rate at the same molt stage. Further, X-radiography demonstrated that barium sulfate ingested by lobsters during ecdysis is accumulated in the midgut just after ecdysis. These results strongly suggest that the midgut is the major surface for water uptake during ecdysis.

Using the in vitro perfusion technique, the ion and water transport properties of the midgut of M. rosenbergii and H. americanus have been

investigated in considerable depth (Ahearn et al., 1977; Ahearn & Tornquist, 1977; Ahearn, 1978; Ahearn, Koosawad & Hadley, 1978; Ahearn, 1980; Wyban, Ahearn & Maginniss, 1980; Mykles, 1981). The findings are summarized as follows. The midgut of M. rosenbergii exhibits a low serosa-positive transmural potential difference (1.6 mV) which is abolished by metabolic inhibitors and ouabain, and dependent upon K^+ concentration, suggesting that maintenance of the potential requires cellular energy and the functioning of (Na^+-K^+) ATPase (Ahearn, 1980). Further, the potential is stimulated by luminal D-glucose and L-alanine but not D-alanine (Wyban et al., 1980). In H. americanus, Mykles (1981) also measured a small serosa-positive potential difference (about 0.26 mV) across the midgut. The potential is dependent upon the presence of Na^+ but not Cl^- in the luminal medium; it is abolished by cyanide but is ouabain-insensitive.

Ahearn et al. (1977) found that transmural fluxes of Na^+ and Cl^- across the midgut of M. rosenbergii are dependent on each other. The mucosal uptake of the two ions are also closely linked. They hypothesized a chemical coupling of Na-Cl cotransport, with a stoichiometry of 2 Na^+ : 1 Cl^- . In a follow-up study, Ahearn & Tornquist (1977) showed that both Na^+ and Cl^- uptakes are sigmoid functions of the corresponding luminal ion concentrations, suggesting an allosteric carrier. Ca^{2+} , which acts as an activator to the carrier, is also cotransported (Ahearn, 1978). Net transmural Na^+ transport is reduced to zero by ouabain and enhanced by alanine (Wyban et al., 1980). In contrast to net Na^+ and Cl^- absorption, there is a net K^+ secretion. Passive permeability measurements revealed a cation selective diffusive pathway (Ahearn, 1980).

By imposing transmural osmotic gradient across the midgut of M. rosenbergii, Ahearn et al. (1978) showed that there is less apparent resistance to osmotic flow from serosa to lumen than in the opposite direction. They ascribed this phenomenon to the unstirred layer effect due to submucosal tissues. The isolated midgut of the marine shrimp, Penaeus marginatus, exhibits a similar rectifying property to osmotic water flow (Ahearn et al., 1978).

Ahearn et al. (1977) showed that net water uptake across the midgut of M. rosenbergii is dependent upon the presence of both Na^+ and Cl^- in the luminal medium, suggesting a solute-linked fluid transport mechanism. In accordance with the standing gradient model of isotonic water transport (Diamond, 1964; Diamond & Bossert, 1967), they proposed that in the freshwater shrimp midgut, water diffuses passively down a local osmotic gradient that presumably is built up in the intercellular spaces by a coupled Na-Cl transport in the mucosal border. A similar model to account for Na^+ -dependent net water transport across the midgut of H. americanus was proposed by Mykles (1981). He suggested that the extensive network of smooth endoplasmic reticulum in the basal portion of the epithelial cells may be the site of the standing osmotic gradient responsible for transmural water absorption.

Nutrient Transport in Crustacean Gut

Earlier studies on nutrient absorption of Crustacea focused on the feeding of nutrients to the animal and subsequently examining the gut epithelia with specific staining techniques. Using this approach, Yonge (1924) found that oil is absorbed by the midgut, and iron by the hepatopancreas of the Norwegian lobster, Nephrops norvegicus. With similar techniques, van Weel (1955) showed that iron and fat are absorbed by the hepatopancreas of the crab, Atya spinipes.

More recent studies on nutrient uptake utilized radioactively labeled nutrients as tracers to indicate absorption. Lehman & Scheer (1956) demonstrated ^{32}P -labeled phosphate uptake by the hepatopancreas of the crab, Hemigrapsus nudus. Dall (1967) reported that the rate of [^{14}C]-glucose uptake in the anterior proventriculus of the shrimp, Metapenaeus bennettiae, is too low to be significant in total nutrient absorption. Thus, he suggested that the hepatopancreas is more important in nutrient uptake. Speck & Urich (1970) found that, in the crayfish, Orconectes limosus, all parts of the gut are capable of absorption of ^{14}C -labeled nutrients, with the hepatopancreas being the most important site. Dall (1981) provided evidence that lipids are absorbed by the hepatopancreas in N. norvegicus. The notion that hepatopancreas is the primary site of nutrient absorption is in accord with the belief of other investigators based mainly on histological observations (see van Weel, 1970; Gibson & Barker, 1979; Dall & Moriarty, 1983 for reviews).

Another approach towards the study of nutrient absorption is the use of in vitro gut preparations. Yonge (1936) used this method to show

that the cuticle is the major determinant in the passage of glucose and fatty acids through the foregut of Homarus americanus. Speck & Urich (1970) reported that the isolated foregut and hindgut of O. limosus can transport nutrients. Physiological mechanisms of nutrient transport by the crustacean gut, however, have not been addressed until the studies by Ahearn and co-workers. Using a slit-gut technique, Ahearn (1974) demonstrated a Na^+ -dependent, saturable glycine transport process in the midgut of the marine shrimp, Penaeus marginatus. The mucosal glycine influx is reduced by metabolic inhibitors, alanine, proline, and ouabain. In a follow-up study, Ahearn (1976) demonstrated that Na^+ influx is also saturable and fluxes of Na^+ and glycine are linked to each other. To account for the difference in coupling ratio of the two substrates in different external concentrations, he proposed a model involving a carrier that can combine with Na^+ and glycine in a number of proportions to form complexes for translocation across the mucosal membrane.

Using the in vitro perfusion technique, Ahearn & Maginiss (1977) showed that glucose transport by the midgut of Macrobrachium rosenbergii is a Na^+ -dependent phlorizin-sensitive saturable process. Further, while the glucose absorbed in the midgut is predominantly in phosphorylated forms, there is a net transmural absorption of glucose per se. Brick & Ahearn (1978) found that lysine uptake by the midgut of M. rosenbergii is through a Na^+ -dependent saturable transport system as well as a non-saturable Na^+ -independent homoexchange mechanism. Alanine uptake was also found to be Na^+ -dependent and the alanine absorbed is rapidly metabolized such that there was no net transmural alanine flux observed (Wyban et al., 1980).

Uptake of ascorbic acid by the same tissue was also reported to be Na^+ -dependent (Ahearn, 1982).

When compared to the mammalian intestine, the midgut nutrient uptake processes of the two crustaceans studied exhibit relatively higher affinities and lower maximal transport rates. In addition, the transmural nutrient fluxes are relatively low. Based on these comparisons, Ahearn (1982) attributed a minor role in nutrient absorption to the crustacean midgut. A recent study using cell suspension techniques showed that alanine transport by the hepatopancreas of Homarus americanus is by means of a Na^+ -dependent saturable process together with an apparent diffusive pathway (Ahearn et al., 1983). The kinetic constants of the saturable component are comparable in magnitude to those reported in mammalian intestine. This study provided further circumstantial evidence that the hepatopancreas, rather than the tubular midgut, is the major site of nutrient uptake. The midgut, with its high-affinity nutrient uptake systems well adapted to the presumably low nutrient levels in its lumen, probably serves a salvage function in absorption.

Scope of the Present Study

From the above literature survey on transport physiology of the crustacean gut, the following general conclusions can be drawn:

1. Many crustaceans ingest their ambient medium. When under osmotic stress, the drinking rate of some species changes in such a direction that this process can be interpreted as adaptive.
2. The gut, or at least part of it, is capable of vectorial ion transport. The role of this route of salt movement in osmotic and ionic regulation is still far from being understood.
3. The midgut is capable of net fluid absorption. This route of water entry contributes significantly towards fluid uptake during ecdysis.
4. Probably all parts of the gut are capable of nutrient uptake. The hepatopancreas is the primary site of absorption with the midgut playing a secondary role.
5. Certain nutrient transport mechanisms in the gut of a few species have been elucidated. A saturable Na^+ -dependent component is involved.

It is apparent that these general conclusions are based on works on a limited number of species and there are still many gaps in our understanding of transport physiology of the crustacean gut. Studies such as those of Ahearn and co-workers are necessary to clarify transport properties of the gut. In the present study, I attempted to investigate the functions of the midgut and hindgut of the blue crab, Callinectes sapidus, with an in vitro perfusion technique. The specific objectives of the present study are:

1. To investigate sodium transport across the midgut as well as the hindgut of Callinectes sapidus and the functioning of these two regions in osmotic and ionic regulation of the animal, and
2. To elucidate the mechanism of glucose absorption across the midgut of C. sapidus and to assess its role in nutrient absorption.

The structure of the gastrointestinal tract of Callinectes sapidus was described by Lochhead (1950) and Pyle & Cronin (1950). Like all other crustaceans, the gut of C. sapidus consists of the foregut, midgut, and hindgut. The foregut is composed of the mouth, a short esophagus, and the cardiac and pyloric stomachs. Numerous calcified ossicles are found in the wall of the cardiac stomach, forming an elaborate grinding apparatus known as the gastric mill. The pyloric stomach contains a press and a complicated filtering apparatus called the gland filter. The midgut is made up of the midgut proper, hepatopancreas, a pair of anterior midgut ceca, and a single posterior midgut cecum. The hepatopancreatic ducts and the tubular anterior midgut ceca enter the pyloric stomach at its junction with the midgut. The straight midgut extends to the junction between the cephalothorax and the abdomen. The tubular, highly coiled posterior midgut cecum empties into the midgut near its end. The hindgut, with conspicuous longitudinal ridges made up of muscle bands, extends from the end of the midgut to the anus.

In the two frequently used references on the anatomy of C. sapidus, the posterior midgut cecum was identified as the hindgut cecum (Lochhead, 1950) or the intestinal cecum (Pyle & Cronin, 1950), implying that it is derived from the hindgut (intestine). This misinterpretation is clearly

due to their erroneous assumption of a short midgut in C. *sapidus*. Similar misconceptions have been made in many brachyurans, and probably arose from an overgeneralization based on the presence of a short midgut in the crayfish. The confusion in the extent of the midgut and the origin of the posterior midgut cecum has been resolved in a number of decapods (Smith, 1978). These anatomical aspects in C. *sapidus* have also been clarified recently (Johnson, 1980).

The ultrastructure of the midgut of Callinectes *sapidus* has been studied by Johnson (1980), whose observations are briefly described here. The midgut epithelium is columnar to almost squamous, depending on the degree of distention. The microvillous apical border is covered by a glycocalyx. The nuclei are generally large, basal in position. One or more large vacuoles may be present in the epithelial cell. A branching tubular network is present in the basal region of the cell. The epithelium is surrounded distally by a thick basement membrane, outside which lie close-set bands of circular muscles. Presence of longitudinal muscular fibers cannot be ascertained.

Due to its large size, ready availability, and ease of maintenance in the laboratory, Callinectes *sapidus* has been a favorite animal for the study of osmoregulation. While being roughly isosmotic in seawater, the animal is an effective hyperosmotic regulator at lower salinities (Tan & Van Engel, 1966; Ballard & Abbott, 1969; Tagatz, 1971; Gerald & Gilles, 1972; Lynch, Webb & Van Engel, 1973; Findley & Stickle, 1978). Like all crustaceans, the blue crab has potentially four major sites of salt and water exchange: gills, excretory organs, integument, and gut. While much

attention has been given to the functioning of the general body surface (Cameron, 1978; Pressley, Graves & Krall, 1981), the gills (King, 1965, 1966; Mantel, 1967; Copeland & Fitzjarrell, 1968; Engel & Eggert, 1974; Engel, Ferguson & Eggert, 1975; Towle, Palmer & Harris, 1976; Cantelmo, 1977; Neufeld, Holliday & Pritchard, 1980; Smith & Linton, 1981; Pressley & Graves, 1983; Salvage & Robinson, 1983) and the antennary glands (Cameron & Batterton, 1978; Robinson, 1982) in osmotic and ionic balance of C. sapidus, few data are available on the ion and water transport properties across its gut.

Gifford (1962) found that whereas the concentrations of Na^+ and Cl^- in the stomach fluid of the blue crab roughly equal to their corresponding hemolymph levels, Mg^{2+} and SO_4^{2-} are more concentrated in the former than in the latter compartment. He suggested that the gut may secrete the two divalent ions into the lumen. However, since the concentrations of the two ions in the stomach fluid are about the same as those of the medium, the simplest explanation of his results is ingestion of the ambient medium. Cantelmo (1977) reported that the gut tissues of C. sapidus acclimated to 40% seawater have a lower apparent water permeability than those acclimated to 100% seawater. In the present study, I have developed an in vitro perfusion technique to investigate Na^+ transport properties across the midgut and hindgut of C. sapidus. To assess their role in osmoregulation, Na^+ fluxes across the tissues were determined in animals acclimated in two dissimilar salinities.

The second part of my dissertation involves the study of glucose transport across the isolated midgut of C. sapidus. To my knowledge, the

nutrient absorptive process in the blue crab has not previously been studied. Glucose was chosen for study because it is the major component of the hemolymph carbohydrates in crustaceans (Hohnke & Scheer, 1970). In C. sapidus, glucose makes up 20-25% of total reducing sugars (Dean & Vernberg, 1965). The seasonal variation of serum glucose level was reported to be 7 to 70 mM (Lynch & Webb, 1973). In the present study, I have investigated transmural transport and mucosal uptake of glucose by the in vitro perfused midgut of C. sapidus. The metabolism of the glucose absorbed was also examined.

CHAPTER II

TRANSMURAL SODIUM TRANSPORT ACROSS THE IN VITRO PERFUSED

MIDGUT AND HINDGUT OF CALLINECTES SAPIDUS

INTRODUCTION

The gastrointestinal tract of Crustacea plays at least two potential physiological roles: nutrient digestion and absorption, and osmotic and ionic regulation. While the former functioning of the crustacean gut has been investigated in considerable depth (see Dall & Moriarty, 1983 for a review), the role of the gut as an organ of salt and water balance remains obscure. For many years, there have been suggestions based on histological and limited physiological evidence that some parts of the gut are responsible for osmoregulation (see Mantel & Farmer, 1983 for a review). Although many studies suggest that the crustacean gut is capable of vectorial salt and water transport, direct physiological investigations on the ion and water transport properties of the gut are scarce. Ahearn et al. (1977) demonstrated net sodium and chloride absorption across the isolated midgut of the freshwater prawn, Macrobrachium rosenbergii. The mechanism of this ion uptake has subsequently been elucidated (Ahearn & Tornquist, 1977; Ahearn, 1978). This route of salt entry may contribute to hyperosmotic regulation of the animal. It is generally believed that the regulatory mechanisms necessary for survival in freshwater habitats derived from modification and hypertrophy of pre-existing physiological mechanisms in non-freshwater precursors (Lockwood, 1977). There has been no equivalent study, however, to demonstrate ion absorption in the gut of marine or estuarine crustaceans, or to investigate its role in osmoregulation.

Many estuarine crustaceans, exposed to daily and seasonal salinity fluctuation, are good osmoregulators. The blue crab, Callinectes

sapidus, maintains its hemolymph isosmotic in seawater above 25 o/oo, and regulates hyperosmotically with respect to the external medium in lower salinities (see Appendix A). Blue crabs in a reduced salinity environment must therefore cope with an osmotic water gain and diffusive salt loss. Excess water entry is excreted through the antennary glands (Cameron & Batterton, 1978; Robinson, 1982), and salt loss is compensated by active absorption of sodium and chloride by the gills (Mantel, 1967; Smith & Linton, 1981). The contribution of the gut in ion uptake has not been investigated.

The present study was undertaken to examine sodium transport across the midgut and hindgut of the blue crab, Callinectes sapidus, with an in vitro perfusion technique. To assess the role of this pathway in osmoregulation, the rate of sodium fluxes across the gut tissues were determined in crabs acclimated to two different salinities. A net sodium absorption was demonstrated across the isolated midgut of the animal. Yet, this net flux does not vary with acclimated salinity, thus providing no evidence for a regulatory role of the midgut in sodium balance of C. sapidus. A reduction of passive sodium permeability with salinity, however, is suggested. Exchange of sodium across the hindgut is too small to be significant in sodium regulation.

MATERIALS AND METHODS

Acclimation of animals

The blue crabs were purchased from Gulf specimen Co., Inc., Panacea, Florida and were air-shipped to Woods Hole, Massachusetts. They were maintained in running seawater at $20 \pm 1^\circ\text{C}$ for 7 d before acclimation experiments. Each animal was fed daily with 4-5 freeze-dried krill. All animals used in this study were at intermolt, usually with a carapace width of greater than 10 cm and a body weight of greater than 100 g.

The animals were acclimated to 34 o/oo and 7 o/oo S in 100 l insulated tanks. Seawater of 7 o/oo was prepared by mixing 34 o/oo seawater with fresh water. The animals were acclimated in a stepwise fashion for 3 d until the final salinity was attained. Then, if necessary, fresh water was added daily to maintain the desired salinity during the acclimation period. The media were aerated and maintained at $20 \pm 1^\circ\text{C}$. Wastes were removed with charcoal filters. Animals were kept in individual cages and fed daily as before. Not more than four crabs were acclimated in a tank at a time. Preliminary experiments showed that at 7 o/oo S, hemolymph osmotic pressure was stabilized after 7 d of acclimation. Thus all animals were acclimated for 7 to 14 d before in vitro perfusion experiments were executed.

Physiological salines

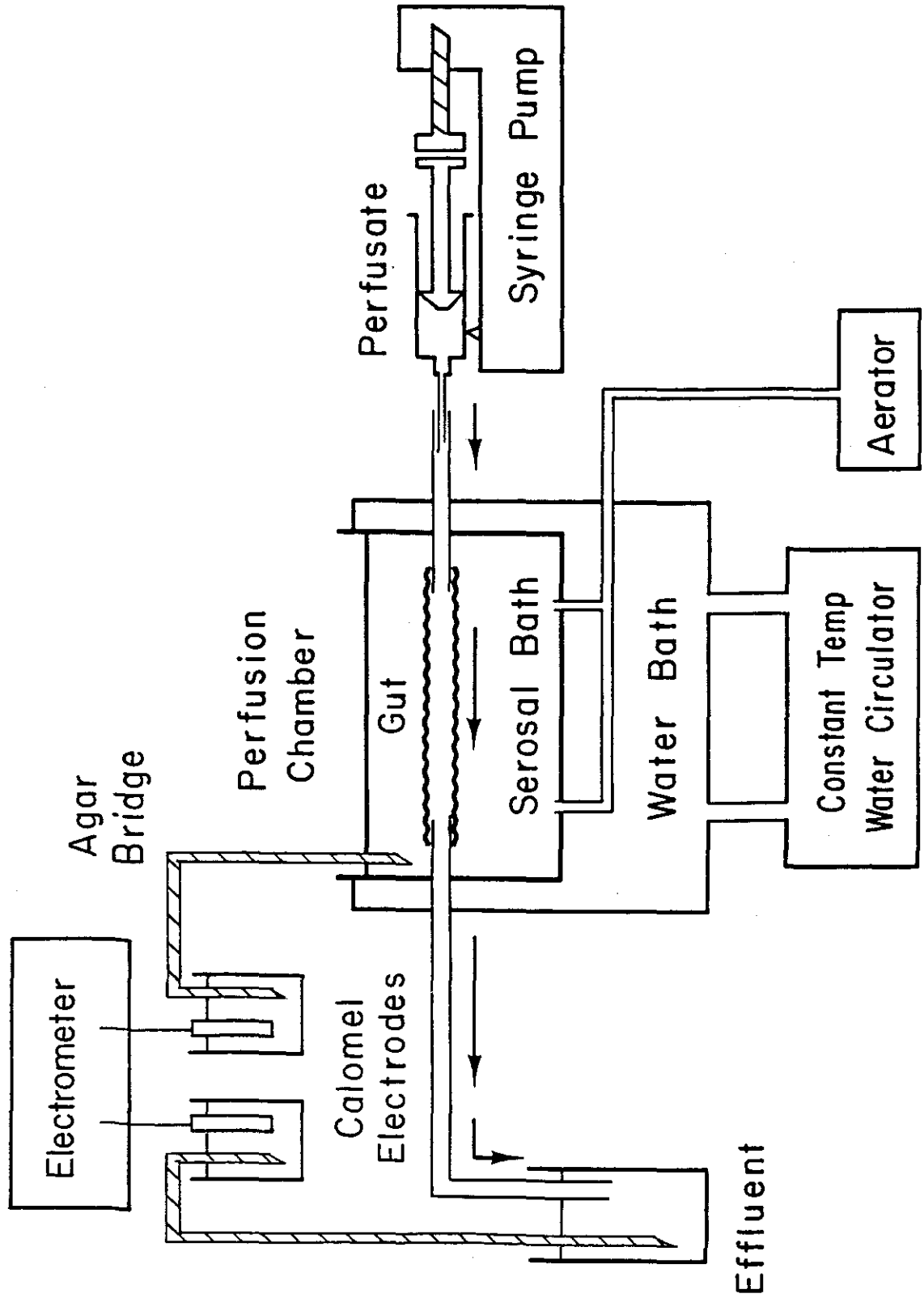
Composition of physiological salines was based on osmotic pressure and ion concentrations of serum determined in animals acclimated at the

two experimental salinities (see Appendix A). For animals acclimated at 34 o/oo S, I used a saline with an osmotic pressure of 960 mosmol kg⁻¹ and a composition as follows: 460 mM NaCl, 11 mM KCl, 13 mM CaCl₂, 13 mM MgSO₄·7H₂O, 50 mM mannitol, and 5 mM HEPES buffer. The saline used for animals acclimated in 7 o/oo S had an osmotic pressure of 660 mosmol kg⁻¹ and contained the following: 300 mM NaCl, 7 mM KCl, 8 mM CaCl₂, 3 mM MgCl₂·6H₂O, 3 mM MgSO₄·7H₂O, and the same amounts of mannitol and HEPES buffer as the above saline. Unless otherwise specified, 5 mM glucose was also included in both salines. Media of various sodium concentrations were prepared by substituting a portion of sodium chloride with an osmotically equivalent amount of recrystallized choline chloride. All salines were prepared with chemicals of reagent grade and adjusted to a pH of 7.5 by adding a few drops of 1 M NaOH solution.

In vitro perfusion experiments

Transmural potential difference and Na⁺ transport across the midgut and hindgut were determined by an in vitro perfusion technique. The midgut or hindgut excised from the animal was flushed with physiological saline and then secured with cotton threads on two catheters, which were either blunt hypodermic needles (18 gauge) or polyethylene tubings (PE 60) in a perfusion chamber (Fig. II-1). The gut mounted in the chamber was usually between 1.5 and 2.5 cm long. The gut was perfused with physiological saline using a syringe pump (Harvard Apparatus). The serosal medium, with a volume of 5 ml, was maintained at 20 ± 1°C and continuously oxygenated and stirred by bubbling with water-saturated air throughout the experimental period.

FIG II-1. Diagram of the apparatus used for in vitro perfusion experiments.



Potential difference across the isolated gut tissue was measured with two 4% agar bridges with salt composition identical to the physiological saline. One agar bridge was placed in the effluent and the other in the serosal bath. They were connected via two calomel electrodes to a Keithley Model 614 digital electrometer. All measurements were corrected for electrode potential asymmetries before the experiment. Due to initial fluctuation of the transmural potential difference, measurements were made 20 min after mounting when the potential had stabilized. It usually remained stable for 1 to 2 h.

The unidirectional transmural Na^+ fluxes across the gut tissue were determined in separate experiments using $^{22}\text{NaCl}$ (New England Nuclear) as the tracer. About 15 min elapsed from the sacrificing of the animal to start of flux experiment, which usually lasted for 90 min and rarely exceeded 150 min. Preliminary experiments showed that change of perfusion rate from 50 to 200 $\mu\text{l min}^{-1}$ had no effect on transmural fluxes. All data reported are from experiments using a perfusion rate of about 100 $\mu\text{l min}^{-1}$. Radioactivity of the tracer was measured with a Beckman LS-100C liquid scintillation counter using Biofluor (New England Nuclear) as the scintillator. All fluxes were normalized to the surface area of the tissue estimated by considering the tubular gut as a cylinder.

To measure unidirectional mucosal to serosal flux, ^{22}Na was added to the perfusate and 0.1 ml aliquots of the serosal medium were taken at 10 min intervals. The volume in the bath was kept constant by replacing each aliquot removed with an equal volume of saline. The appearance of radioactivity in the bath was linear with time after the first 10 min and

remained constant for 2 h. The flux was computed from the slope of the linear regression line of the increase of ^{22}Na activity with time.

To measure unidirectional serosal to mucosal flux, ^{22}Na was added to the bath and equal volumes of effluent were collected each for every 5 or 10 min. The activity of ^{22}Na in the effluent was stabilized after the first 20 min. The flux was calculated from the mean activity of five or more samples after this initial time period.

In some experiments, both unidirectional fluxes across the midgut were determined in a single tissue by flushing the gut clear of radioactivity with saline between the two flux periods. Transmural potential difference was monitored throughout the experiment.

In a series of experiments, change of unidirectional mucosal to serosal Na^+ flux across the midgut with mucosal Na^+ concentration was investigated by perfusing the gut sequentially with salines of increasing Na^+ concentration and equal specific radioactivity. The concentrations used were 50, 150, 250, 350, and 460 mM. Na^+ concentration in the serosal bath was maintained constant at 460 mM. In another series of experiments, variation of unidirectional serosal to mucosal flux with serosal Na^+ concentration was studied by exposing the tissue sequentially to salines of the above Na^+ concentrations and keeping the concentration in the perfusing saline at 460 mM.

The effect of potassium cyanide or ouabain on unidirectional mucosal to serosal Na^+ flux across the midgut was investigated by adding the inhibitor to the serosal bath after a control flux period of 40 min. After 10 min of incubation, the flux was determined for another 40 min.

The effect of glucose was studied in a similar manner, by determining the flux across the midgut perfused with glucose-free saline before or after the control flux period.

Measurement of oxygen consumption rates (Q_{O_2}) of isolated midgut and hindgut using a Gilson differential respirometer showed that these tissues could maintain a stable rate of Q_{O_2} for more than 2 h after isolation from the animal (see Appendix B). Moreover, the rate was abolished in response to cyanide. This property, together with the observation of peristaltic and antiperistaltic waves in the gut tissues mounted in the perfusion chamber, indicated that the viability of the gut tissues was maintained during the in vitro perfusion experiments.

Statistical analysis

Data are expressed as mean \pm SEM (n = number of experiments). Mean values were compared using paired or unpaired Student's t-test. In unpaired tests, if the variance ratio indicated unequal variances, a modified t-test was used instead (Baily, 1959). Significant difference between mean values was assumed at a probability level of 0.05.

RESULTS

Transmural potential difference

The isolated midgut and hindgut from animals acclimated at 34 o/oo S exhibited serosa-positive transmural potential differences of 1.4 ± 0.4 mV (12) and 1.8 ± 0.2 mV (12), respectively. The two values are significantly different from zero but are not different from each other. Further, they are not significantly different from the potentials measured in animals acclimated at 7 o/oo S. The corresponding values in the lower experimental salinity are 1.3 ± 0.3 mV (6) in the midgut and 1.6 ± 0.2 mV (6) in the hindgut.

Transmural sodium transport

The transmural Na^+ fluxes across the isolated midgut and hindgut in crabs acclimated at 34 o/oo and 7 o/oo S are shown in Table II-1. In both experimental salinities, the mucosal to serosal flux, J_{ms} , across the midgut significantly exceeds the serosal to mucosal flux, J_{sm} . The resulting net fluxes, J_{net} , are significantly greater than zero. The two net fluxes across the midgut are not significantly different from each other.

The effects of potassium cyanide, ouabain, and the depletion of glucose on unidirectional mucosal to serosal Na^+ flux across the midgut in crabs acclimated at 34 o/oo S are shown in Fig. II-2. While 1 mM potassium cyanide had no apparent effect on the flux, 0.5 mM ouabain reduced it by 20%. The flux was not significantly affected by the depletion of glucose in the luminal medium.

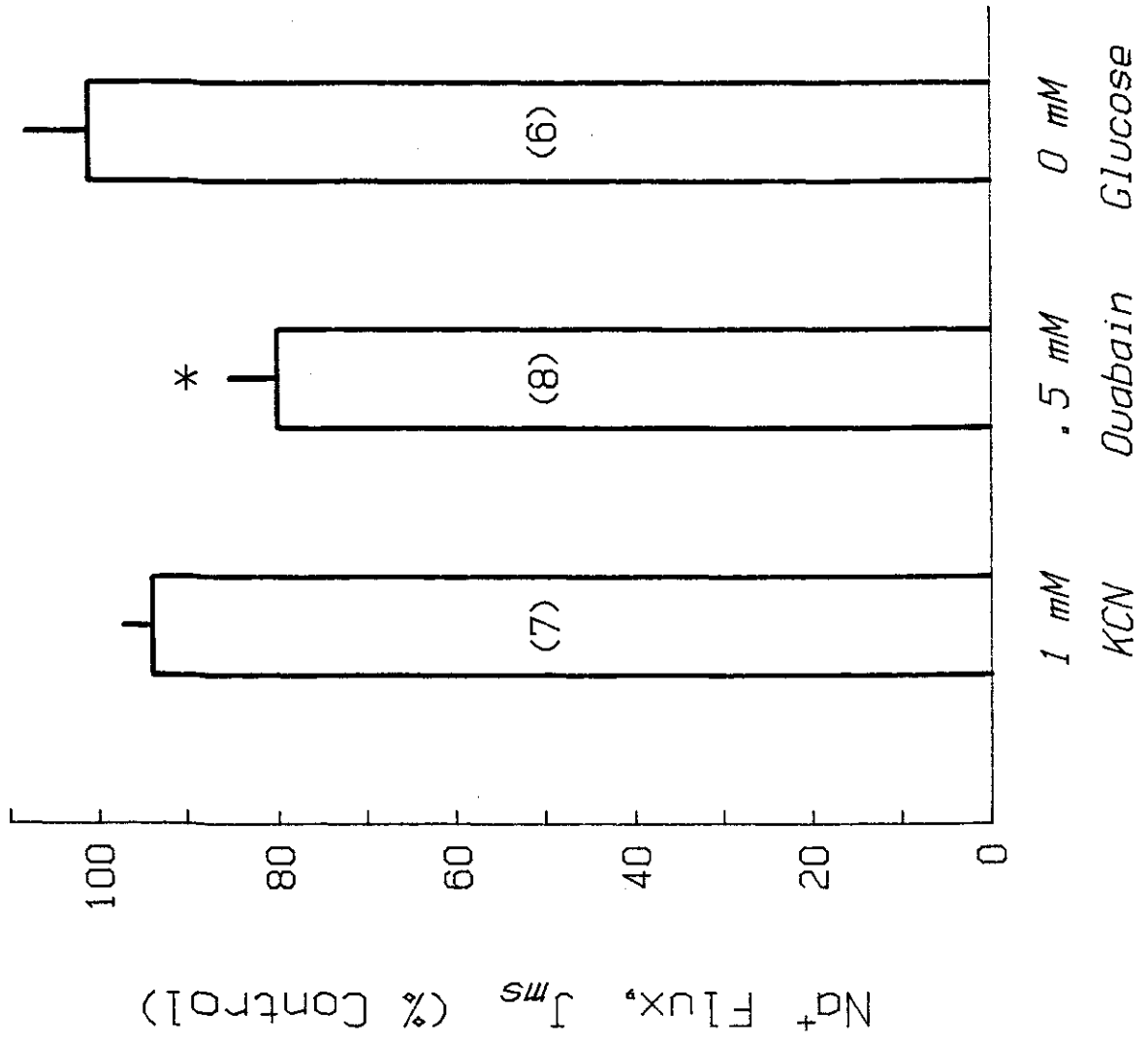
TABLE II-1

Transmural Na⁺ fluxes across the isolated midgut and hindgut of Callinectes sapidus acclimated at 34 o/oo and 7 o/oo S

	Acclimation salinity (o/oo)	Transmural Na ⁺ fluxes ($\mu\text{mol cm}^{-2} \text{ h}^{-1}$)		
		J _{ms}	J _{sm}	J _{net}
Midgut	34	15.5 \pm 2.2 (18)	10.4 \pm 0.5 (6)	5.1 \pm 2.2 *
	7	8.4 \pm 1.5 (18)	5.3 \pm 0.3 (6)	3.1 \pm 1.6 *
Hindgut	34	0.7 \pm 0.3 (10)	0.4 \pm 0.1 (6)	0.3 \pm 0.3
	7	1.0 \pm 0.3 (15)	0.6 \pm 0.2 (7)	0.4 \pm 0.3

Values are means \pm SEM (no. of experiments). J_{ms} and J_{sm} are unidirectional mucosal to serosal and serosal to mucosal fluxes, respectively. Their difference gives the net flux, J_{net}. An asterisk indicates a net flux significantly greater than zero.

FIG. II-2. Effects of potassium cyanide, ouabain, and glucose on unidirectional mucosal to serosal Na^+ flux across the isolated midgut of Callinectes sapidus. Results are expressed as mean % of initial flux (i.e., before treatment), and vertical lines represent 1 SEM. Numbers of experiments are indicated in parentheses. An asterisk indicates a flux after the treatment significantly different from the initial flux.



Na^+ fluxes across the hindgut were markedly lower than those in the midgut (Table II-1). In both acclimated salinities, J_{ms} is not significantly different from J_{sm} resulting in no significant J_{net} across the hindgut.

Since the hindgut is lined with a cuticle, the following experiment was done to test whether the cuticle is the permeability barrier to limit Na^+ movement. The unidirectional mucosal to serosal Na^+ flux was measured in control conditions for 50 min and the serosal medium was then replaced with distilled water to lyse the cells in the mounted tissue. After 10 min, the bath was replenished with saline and the flux was measured for another 50 min. This experiment, performed 5 times, gave J_{ms} values of $0.6 \pm 0.3 \mu\text{mol cm}^{-2} \text{ h}^{-1}$ before and $0.9 \pm 0.4 \mu\text{mol cm}^{-2} \text{ h}^{-1}$ after the treatment. The difference in J_{ms} is marginally significant ($.02 < P < .05$), but the fluxes after cell lysis remained low, indicating that the major barrier to Na^+ movement in the hindgut is the chitinous lining.

Flux ratio analysis of sodium transport across the midgut

Since the above data showed that a net mucosal to serosal Na^+ flux occurs across the midgut in the absence of a concentration gradient and against an electrical gradient imposed by a serosa-positive potential difference, it was suspected that Na^+ flux is mediated by a mechanism other than simple diffusion. This property was further investigated in experiments where unidirectional Na^+ fluxes were measured consecutively in a single midgut preparation with potential difference monitored throughout the experiment. Assuming that Na^+ fluxes are governed solely

by the transmural potential difference, V_m , the ratio of the two unidirectional fluxes is given by the equation (Ussing, 1949):

$$\frac{J_{ms}}{J_{sm}} = \exp \left[-\frac{zF}{RT} V_m \right]$$

where z denotes the charge of Na^+ , F the Faraday constant, R the gas constant, and T the absolute temperature. The experimental results are shown in Table II-2. In control conditions, the observed fluxes gave a ratio that was significantly greater than the predicted ratio, confirming the notion that net Na^+ transport, at least part of it, is mediated by an active mechanism. Incubating the tissue in 0.5 mM ouabain before flux determinations resulted in no significant net flux measured across the midgut as well as no significant difference between the calculated and experimental flux ratios, indicating that bidirectional fluxes in the presence of ouabain are adequately explained by simple diffusion.

Transmural sodium fluxes across the midgut as a function of $[\text{Na}^+]$

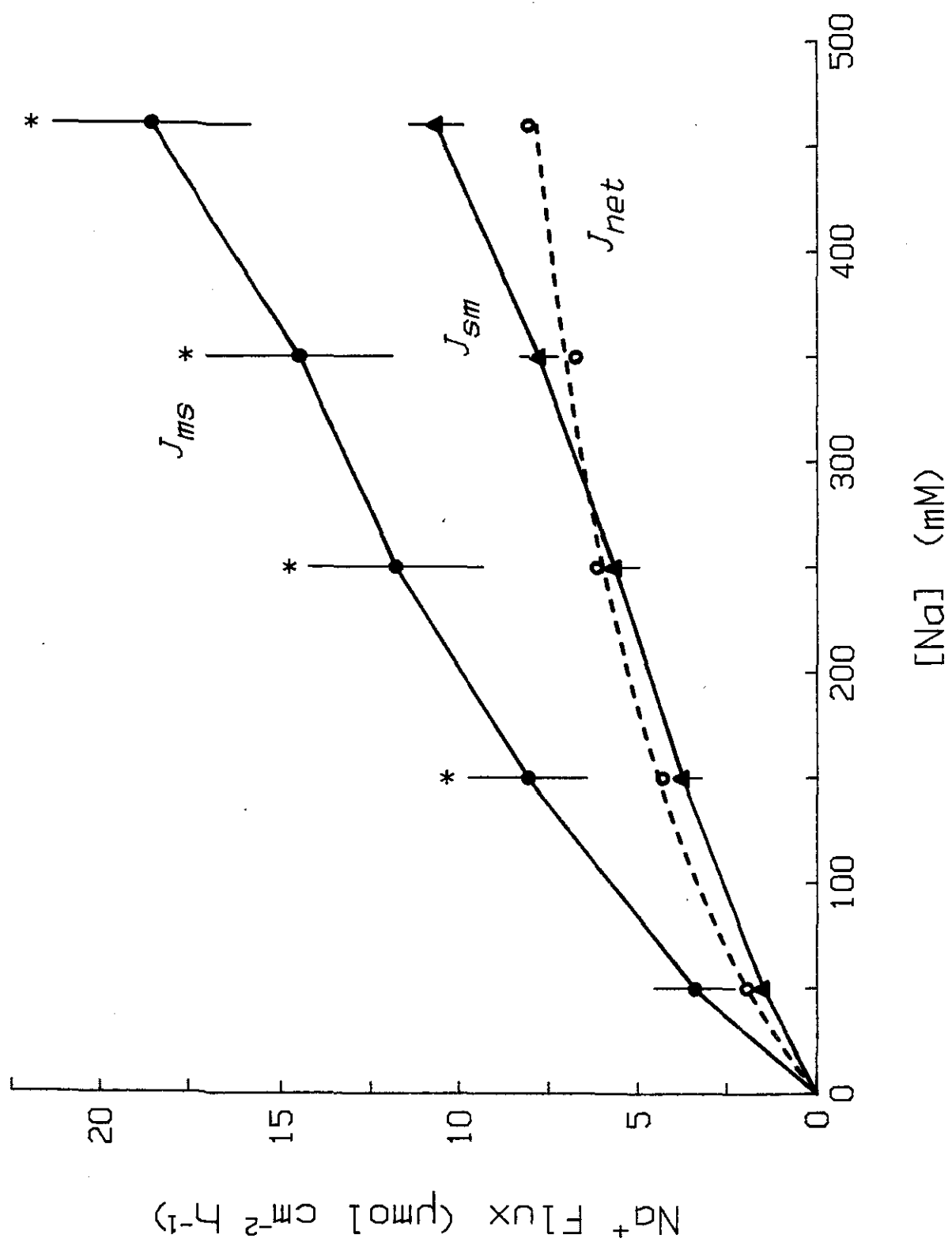
Unidirectional mucosal to serosal flux across the midgut appeared to be a curvilinear function of mucosal Na^+ concentration, as shown in Fig. II-3. Variation of the flux in the opposite direction with serosal Na^+ concentration was essentially linear ($r^2 = 0.91$), with intercepts that are not significantly different from zero. Its proportionality with external concentration indicates that J_{sm} is through a diffusive pathway. The apparent diffusion coefficient is estimated to be $21.7 \text{ nmol cm}^{-2} \text{ h}^{-1} / \text{mM} [\text{Na}]$. J_{ms} is significantly greater than J_{sm} except at the lowest

TABLE II-2.
Transmural Na^+ fluxes, potential difference, and observed and predicted flux ratios
across the isolated midgut of Callinectes sapidus in the presence and absence of ouabain

	N	Transmural Na^+ fluxes ($\mu\text{mol cm}^{-2} \text{ h}^{-1}$)			Transmural potential difference		Flux ratio	
		J_{ms}	J_{sm}	J_{net}	V_m (mV)	observed J_{ms}/J_{sm}	predicted $\exp[-zFV_m/RT]$	
Control	4	14.9 ± 2.0	8.3 ± 0.8	6.6 ± 2.3	2.9 ± 0.9	1.87 $\pm .38$	0.89 ± 0.03	*
0.1 mM ouabain	3	6.3 ± 1.1	6.6 ± 1.0	- 0.3 ± 1.1	1.4 ± 0.6	0.97 ± 0.16	0.95 ± 0.04	

Values are means \pm SEM. N is the number of experiments. An asterisk indicates an observed ratio significantly greater than the corresponding predicted ratio.

FIG. II-3. Variation of transmural Na^+ fluxes across the isolated midgut of Callinectes sapidus with Na^+ concentration. Data points of unidirectional fluxes from mucosal to serosal, J_{ms} (closed circles), and from serosal to mucosal, J_{sm} (triangles), are the mean of 4 experiments, and the bars indicate \pm SEM. In some cases, half of the bar is omitted for clarity. When no bars are shown, they are within the region of the symbol. The difference between the two fluxes gives the net flux, J_{net} (open circles). The dashed line representing J_{net} was computed from the Michaelis-Menten equation using the kinetic constants derived from the single reciprocal plot illustrated in Fig. II-4.

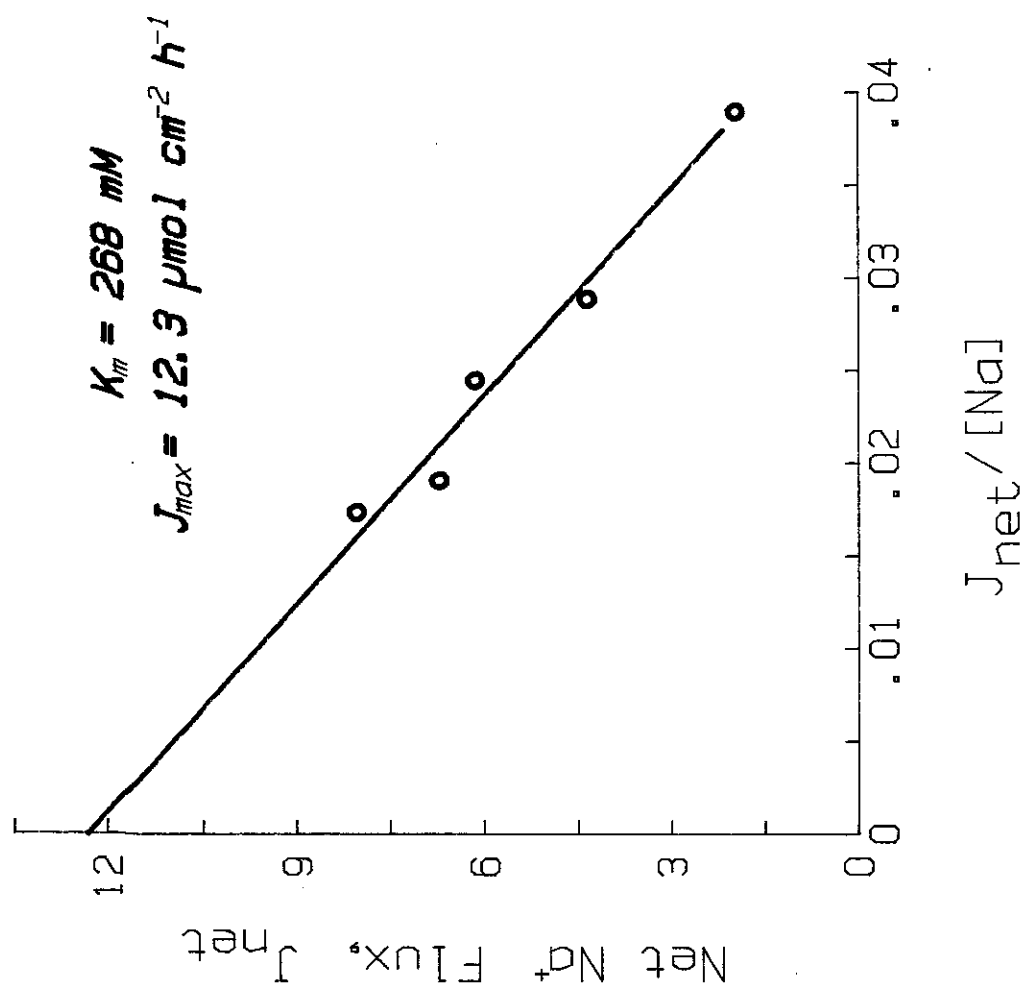


experimental $[\text{Na}^+]$ of 50 mM. Net Na^+ transport, J_{net} , is taken as the difference between the corresponding J_{ms} and J_{sm} . A single reciprocal plot of J_{net} vs. $J_{\text{net}}/[\text{Na}]$, as illustrated in Fig. II-4, shows that it is a saturable flux, which can be represented by the Michaelis-Menten equation:

$$J_{\text{net}} = \frac{J_{\text{max}}[\text{Na}]}{K_{\text{m}} + [\text{Na}]}$$

where J_{max} is the flux at saturation, and K_{m} is the $[\text{Na}^+]$ where $J_{\text{net}} = J_{\text{max}}/2$. The plot gives a K_{m} of 268 mM and a J_{max} of $12.3 \mu\text{mol cm}^{-2} \text{ h}^{-1}$. A similar saturable flux can be computed by assuming that J_{ms} is made up of saturable and non-saturable components, and then subtracting the latter from the total flux. In this case, the linear non-saturable component coincides with J_{sm} , indicating that this portion of J_{ms} goes through the same route as J_{sm} , probably the paracellular pathway.

FIG. II-4. Single reciprocal plot of net transmural Na^+ flux, J_{net} vs. $J_{\text{net}}/[\text{Na}]$. K_m was determined from the slope and J_{max} from the vertical intercept of the regression line ($r^2 = 0.97$).



DISCUSSION

Characteristics of sodium transport across the midgut and hindgut

The present study demonstrates a net Na^+ absorption across the in vitro perfused midgut of Callinectes sapidus. Unidirectional flux ratio analysis and the saturable nature of the net Na^+ flux strongly suggest that it is an active transport process. The net Na^+ flux determined across this tissue is within the same order of magnitude to Na^+ fluxes reported in the guts of mammals (Schultz & Zalusky, 1964a; Al-Awqati, Cameron & Greenough, 1973), fish (Oide, 1967; Utida et al., 1972; Huang & Chen, 1971; Smith, Ellory & Lahlou, 1975; Field et al., 1978), the bullfrog (Quay & Armstrong, 1969), the gastropod Aplysia (Gerencser & Hong, 1977; Gerencser, 1978) and the freshwater shrimp, Macrobrachium rosenbergii (Ahearn et al., 1977). This net Na^+ transport across the midgut of C. sapidus may be partially responsible for the low serosa-positive potential difference observed in this tissue. Transmural potential difference of the same polarity and magnitude is also exhibited by the midgut of M. rosenbergii (Ahearn, 1980), and the American lobster, Homarus americanus (Mykles, 1981).

Like many other animal tissues capable of active Na^+ transport, the unidirectional mucosal to serosal flux across the midgut of C. sapidus is ouabain-sensitive, suggesting that the functioning of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ is involved in transmural Na^+ absorption. This flux, however, is not inhibited by potassium cyanide, indicating that the energy required for the transport is not derived from oxidative metabolism. An anaerobic pathway

is probably responsible for this energy resource. Moreover, J_{ms} is not affected by the absence of luminal glucose. The stimulation of transmural Na^+ transport by glucose has been reported in mammalian (Curran, 1960; Schultz & Zalusky, 1964b; Taylor et al., 1968, Barry, Eggenton & Smyth, 1969) and invertebrate (Gerencser & Hong, 1977; Gerencser, 1978) guts. In mammalian studies, this increase in Na^+ transport is often attributed to the functioning of a Na^+ -glucose cotransporter in the mucosal border of the intestine (Goldner, Schultz & Curran, 1969). It has been shown that Na^+ -dependent glucose uptake is present in the midgut of C. sapidus (Chu, 1983; see Chapters III & IV). The glucose flux in this tissue, however, is so low that, assuming a low Na^+ : glucose coupling ratio, the increase in Na^+ flux through this pathway is minimal.

The hindgut of the blue crab exhibits a low serosa-positive transmural potential difference similar to that of the midgut. In contrast to the midgut, however, the Na^+ fluxes across the hindgut are low and no net transmural transport could be demonstrated. Malley (1977b) also reported limited Na^+ movement across the hindgut of the spiny lobster, Panulirus argus. The difference in Na^+ transport properties between these two regions of the gut in C. sapidus is probably due to the presence of a chitinous lining in the hindgut. Yonge (1936) also showed that the cuticle is the major permeability barrier in the foregut of H. americanus.

Role of sodium transport across the gut in Na⁺ hyperegulation

Callinectes sapidus regulates its hemolymph Na⁺ level higher than that of the ambient seawater in low salinity environments (Mantel, 1967; Lynch, Webb & Van Engel, 1973; Findley & Stickle, 1978; see also Appendix I). It has been shown that the gills are the site of active Na⁺ absorption in dilute salinities (Mantel, 1967; Smith & Linton, 1971). Under these conditions, respiratory rate and activity of (Na⁺-K⁺)-ATPase of the gills increase (King, 1965, 1966; Engel & Eggert, 1974; Engel, Ferguson & Eggert, 1975; Towle, Palmer & Harris, 1976; Neufeld, Holliday & Pritchard, 1980; Savage & Robinson, 1983). An intention of the present study is to investigate the possible role of the midgut and hindgut in the Na⁺ regulatory process.

Table II-1 shows that the unidirectional Na⁺ fluxes across the hindgut of the blue crab are usually under 1 $\mu\text{mol cm}^{-2} \text{ h}^{-1}$. Total Na⁺ fluxes in the whole crab are large, averaging 600-700 $\mu\text{mol 100 g}^{-1} \text{ h}^{-1}$ in animals living in fresh water (Cameron, 1978). Since Na⁺ transport in the hindgut only contributes a minor portion of the total Na⁺ exchange, the functioning of this region of the gut in hemolymph Na⁺ regulation is negligible. Malley (1977b) also ascribed a minor role of the hindgut in osmotic and ionic balance in Panulirus argus.

In the midgut of C. sapidus, a net Na⁺ absorption of about 5 $\mu\text{mol cm}^{-2} \text{ h}^{-1}$ is evident. Measurement of this route of Na⁺ entry at a lower acclimated salinity, however, provides no evidence for the capability of the midgut to increase its rate of Na⁺ absorption in response to external salinity. Mantel (personal communication) found that in the same tissue,

the activity of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$, an enzyme believed to play an important role in osmotic and ionic regulation (see Towle, 1981 for a review), is not affected by acclimated salinity. This finding provides additional evidence that active Na^+ absorption by the midgut is of limited importance in Na^+ hyperegulation.

It should be emphasized, however, that osmoregulation in many crustaceans is under neuroendocrine control (see Kamemoto, 1976; Mantel, 1984 for reviews). With respect to gut transport, Mantel (1968) found that ion and water permeability of the isolated foregut of the land crab, Gecarcinus lateralis is regulated by extracts from the thoracic ganglionic mass. Kamemoto (1976) also reported that neuroendocrine factors influence water movement in the isolated hindgut of the crayfish, Procambarus clarkii. Thus, in my study using isolated tissues, it is possible that only basal Na^+ fluxes were measured and any changes mediated by neuroendocrine factors in response to salinity acclimation might escape detection.

Although the present study fails to illustrate any osmoregulatory role of net Na^+ transport across the midgut, some of my data indicate that the passive permeability of the tissue may be involved in Na^+ balance. Assuming that the unidirectional serosal to mucosal flux determined in this study is predominantly through the paracellular pathway, I can calculate the passive Na^+ permeability through this pathway by using the Goldman constant field equation:

$$J_{sm} = - P_{Na} \frac{zFV_m/RT}{1 - \exp(zFV_m/RT)} [\text{Na}]_s \exp(zFV_m/RT)$$

where P_{Na} is the apparent Na^+ permeability, $[Na^+]_s$ the sodium concentration in the serosal medium, and other parameters were defined before. Using the corresponding experimental values of J_{sm} , V_m , and $[Na]_s$ in animals acclimated to 34 o/oo and 7 o/oo S, the two permeability coefficients in the midgut are estimated to be 6.1 ± 0.3 (6) and 4.8 ± 0.3 (6) $\times 10^{-6} \text{ cm s}^{-1}$, respectively. The two values are significantly different from each other, indicating that the midgut of animals acclimated at a lower salinity reduces its passive Na^+ permeability.

In my in vitro experiments, the Na^+ concentration on both sides of the gut was kept at the same level as that of the hemolymph. In in vivo conditions, when Callinectes sapidus enters waters of lower salinities, the midgut lumen may have a Na^+ concentration much lower than that of the hemolymph due to ingestion of the ambient medium. Thus, instead of a net absorption through the midgut as determined in the in vitro experiments, a net loss of Na^+ may take place. Assuming that in spite of a change in the direction of net flux, the alteration of passive Na^+ permeability remains as determined above, a reduction of the permeability in lower salinity would minimize Na^+ exchange through the midgut. This mechanism will not only assist in slowing fluctuation of hemolymph Na^+ concentration but also reduce the energy expenditure of compensatory active Na^+ uptake. It has been shown that freshwater and estuarine crustaceans have lower Na^+ loss rates than related marine species, and as some estuarine species go from concentrated to dilute medium, their permeability to water and ions decreases (see Prosser, 1973; Lockwood 1977; Mantel & Farmer, 1983 for reviews). Cantelmo (1977) found that the gill and gut tissue of C.

sapidus reduces their water permeability on acclimation to lower salinities. My data indicate that a similar reduction in the Na^+ permeability may occur in the midgut. The mechanism responsible for this decrease is unclear. It may involve an actual change in permeability of the paracellular pathway including the cellular junction, or an alteration in the geometry of the tissue in response to the decrease of osmotic pressure of its bathing fluids. Because of the large amount of Na^+ exchange across the whole crab (Cameron, 1978) and the small surface area of the tubular midgut, the benefit of a decrease in Na^+ permeability in this tissue towards the overall sodium balance of the animal is probably limited. This decrease, however, may reflect a general reduction in permeability across the epithelia of midgut appendages, including the hepatopancreas. Given the large surface area of these tissues, such a permeability change can contribute substantially towards hemolymph Na^+ regulation of the animal.

CHAPTER III

GLUCOSE TRANSPORT BY THE IN VITRO PERFUSED MIDGUT

OF CALLINECTES SAPIDUS

A. TRANSMURAL FLUXES

INTRODUCTION

Nutrient absorption is an important function of the gut of animals. The mechanisms of nutrient absorption in mammalian intestine have been extensively investigated (see Code, 1968 for review). These studies led to the formulation of the well-known Na^+ -gradient hypothesis (see Schultz & Curran, 1970; Crane, 1977; Schultz, 1977 for reviews). Relatively little is known concerning the nutrient absorptive processes in the gut of invertebrates. In crustaceans, the foregut and hindgut, lined with a cuticle, are generally considered to be of little importance in nutrient uptake. The principal site of absorption is believed not to be in the tubular midgut but in the hepatopancreas (see Gibson & Barker, 1979; Dall & Moriarty, 1983 for recent reviews). During the last decade, a number of reports on nutrient transport mechanisms in the crustacean midgut have appeared (Ahearn, 1974, 1976; Ahearn & Maginniss, 1977; Brick & Ahearn, 1978; see Ahearn, 1982 for a review). These studies showed that the crustacean midgut exhibits many of the basic transport characteristics of mammalian intestine, such as Na^+ -dependency and saturation behavior. Kinetic evidence, however, indicated that the midgut does not play a major role in nutrient uptake. There have been no other nutrient transport studies on crustacean midgut.

The present study deals with glucose transport across the midgut of the common blue crab, Callinectes sapidus. The results indicate that net glucose transport in this tissue is an active, Na^+ -dependent

process. It displays saturation kinetics typical of carrier-mediated transport. As in mammalian intestine, transport of glucose is inhibited by phlorizin. These findings are compatible with the Na^+ -gradient hypothesis. Further, a low rate of glucose absorption supports the notion that the midgut is of minor importance with respect to nutrient absorption.

Preliminary results from this investigation have been reported previously (Chu, 1983).

MATERIALS AND METHODS

Maintenance of animals

Blue crabs were purchased from Gulf Specimen Co., Inc., Panacea, Florida and were maintained in running seawater at $20 \pm 1^\circ\text{C}$ for 7 to 10 d before experimentation. Each animal was kept in an individual cage and fed daily with 4-5 freeze-dried krill. All animals used in the present study were intermolt individuals, usually with a carapace width of greater than 10 cm and a body weight of greater than 100 g.

Physiological saline

The composition of physiological saline used in this study was based on analyses of osmotic pressure and ionic concentrations of hemolymph of the animal. The saline contained the following: 460 mM NaCl, 11 mM KCl, 13 mM CaCl_2 , 13 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM mannitol and 5 mM HEPES buffer; with an osmotic pressure of $960 \text{ mosmol kg}^{-1}$. It also included 1 to 5 mM glucose. The saline was titrated to a pH of 7.5 with a few drops of 1 M NaOH solution. Sodium-free saline was prepared by substituting sodium chloride with an osmotically equivalent amount of recrystallized choline chloride. The saline was adjusted to the same pH by adding KOH solution.

Determination of transmural glucose flux

The in vitro perfusion technique used for this study has been described in Chapter II. Unidirectional transmural glucose fluxes were

determined in separate pieces of tissues with [6-³H]-D-glucose (New England Nuclear) as the tracer. Experimental procedures were similar to those described on the determination of transmural Na⁺ fluxes (Chapter II). Preliminary experiments showed that transmural glucose fluxes were not affected by variation of perfusion rate from 50 to 150 $\mu\text{l min}^{-1}$. All experiments reported here were done at a perfusion rate of 100 $\mu\text{l min}^{-1}$. Tritiated activity was measured with a Beckman LS-100C liquid scintillation counter using Aquasol (New England Nuclear) as the scintillator.

In some experiments determining unidirectional mucosal to serosal flux, two aliquots of serosal medium were sampled. One was counted as described and the other was first evaporated to dryness, then redissolved in the same volume of saline and counted. This procedure was used to estimate the flux of non-volatile forms of glucose through the midgut, by eliminating any tritiated water which may have resulted from metabolism by the tissue.

In a series of experiments, change of unidirectional mucosal to serosal glucose flux across the midgut with mucosal glucose concentration was investigated by perfusing the gut sequentially with salines of increasing glucose concentration and equal specific radioactivity. The glucose concentrations used were 0.5, 1.0, 2.5, and 5.0 mM. Glucose concentration in the serosal medium was maintained constant at 5.0 mM. In another series of experiments, variation of unidirectional serosal to mucosal flux with serosal glucose concentration was studied by exposing the tissue sequentially to salines of the above glucose concentrations in

the serosal bath and keeping the concentration in the perfusing medium at 5.0 mM.

To investigate the effects of inhibitors and hexoses on unidirectional mucosal to serosal transport, the flux was determined in control conditions for 50 min and then the compound of interest was added to the perfusate or the serosal medium. After 10 min of incubation, sampling was resumed as before for another 50 min. The effect of Na^+ was studied in a similar manner, by determining flux across midgut perfused with sodium-free saline before or after the control flux period.

In some experiments, unidirectional transmural glucose fluxes across the midgut were measured simultaneously by double labeling, with $[6\text{-}^3\text{H}]\text{-D-glucose}$ and $[^{14}\text{C(U)}]\text{-D-glucose}$ added to the perfusate and bath, respectively. Radioactivity of samples from the mucosal and serosal media was corrected for quenching with automated external standardization to obtain disintegrations per min. Spillover of ^{14}C activity to the ^3H window could then be corrected accordingly and the fluxes were computed as before.

All fluxes were expressed in nmoles of glucose per cm^2 tissue per hour, by considering the tubular midgut as a cylinder.

Thin layer chromatography

In a few experiments determining mucosal to serosal flux, the chemical state of tritiated activity in the serosal medium was investigated with one-dimensional ascending thin layer chromatography. As a high salt content interfered with chromatographic development, the salts were first precipitated in 95% ice-cold ethanol and spun down in a

clinical centrifuge. The supernatant was concentrated by mild heating and the treatment was repeated three times. The ethanol extract was then spotted on a microcrystalline cellulose plate ("Avicel", Anatech, Inc.). The plate was developed in pyridine-ethyl acetate-acetic acid-water (5: 5: 1: 3) at room temperature (Wolf from, Patin & de Lederkremer, 1965). The solvent was allowed to ascend for 10 cm and the plate was then air-dried. Along a single separation path, segments of 0.5 by 1.5 cm were scraped from the plate, each placed in a scintillation vial, and broken up in 4 ml of distilled water with sonication. Then, 10 ml of Aquasol was added for scintillation counting. A standard of [^3H]-glucose was analysed together with the samples and the R_f of unlabeled glucose was also resolved by spraying with aniline diphenylamine reagent (Sigma).

Statistical analysis

Mean values of flux were compared using paired or unpaired Student's t-test. In unpaired tests, if the variance ratio indicated unequal variances, a modified t-test was used instead (Baily, 1959). Significant difference between mean values was assumed at a probability level of 0.05.

RESULTS

Transmural glucose fluxes

Results of unidirectional transmural glucose fluxes are shown in Table III-1. With separate pieces of isolated midgut and 5 mM glucose on both sides of the tissue, the mucosal to serosal flux, J_{ms} , is significantly greater than the flux in the reverse direction, J_{sm} . The difference between the two gives a significant net flux, J_{net} , of $40 \text{ nmol cm}^{-2} \text{ h}^{-1}$. This finding of a net glucose absorption across the midgut is supported by simultaneous determination of the two fluxes by double labeling experiments carried out in identical glucose concentration. These experiments resulted in a net flux not significantly different from the one determined above. In experiments where the glucose concentration in the luminal medium was lowered to 2.5 mM and that of the serosal medium maintained at 5.0 mM, there was still a net mucosal to serosal glucose flux observed. The net flux is not significantly different from that determined with 5.0 mM glucose concentration on either side of the gut ($P = .084$). This finding indicates that net glucose absorption can take place against a concentration gradient and that a luminal glucose concentration of 2.5 mM is probably large enough to saturate the transport mechanism (see next section).

Thin layer chromatographic analysis of the serosal medium from J_{ms} experiments resulted in a single peak with a R_f indistinguishable from a R_f of 0.48 exhibited by the glucose standard (Fig. III-1). In experiments to determine J_{ms} where the samples from the serosal medium were evaporated to dryness before counting, a reduction of 10 to 20% of

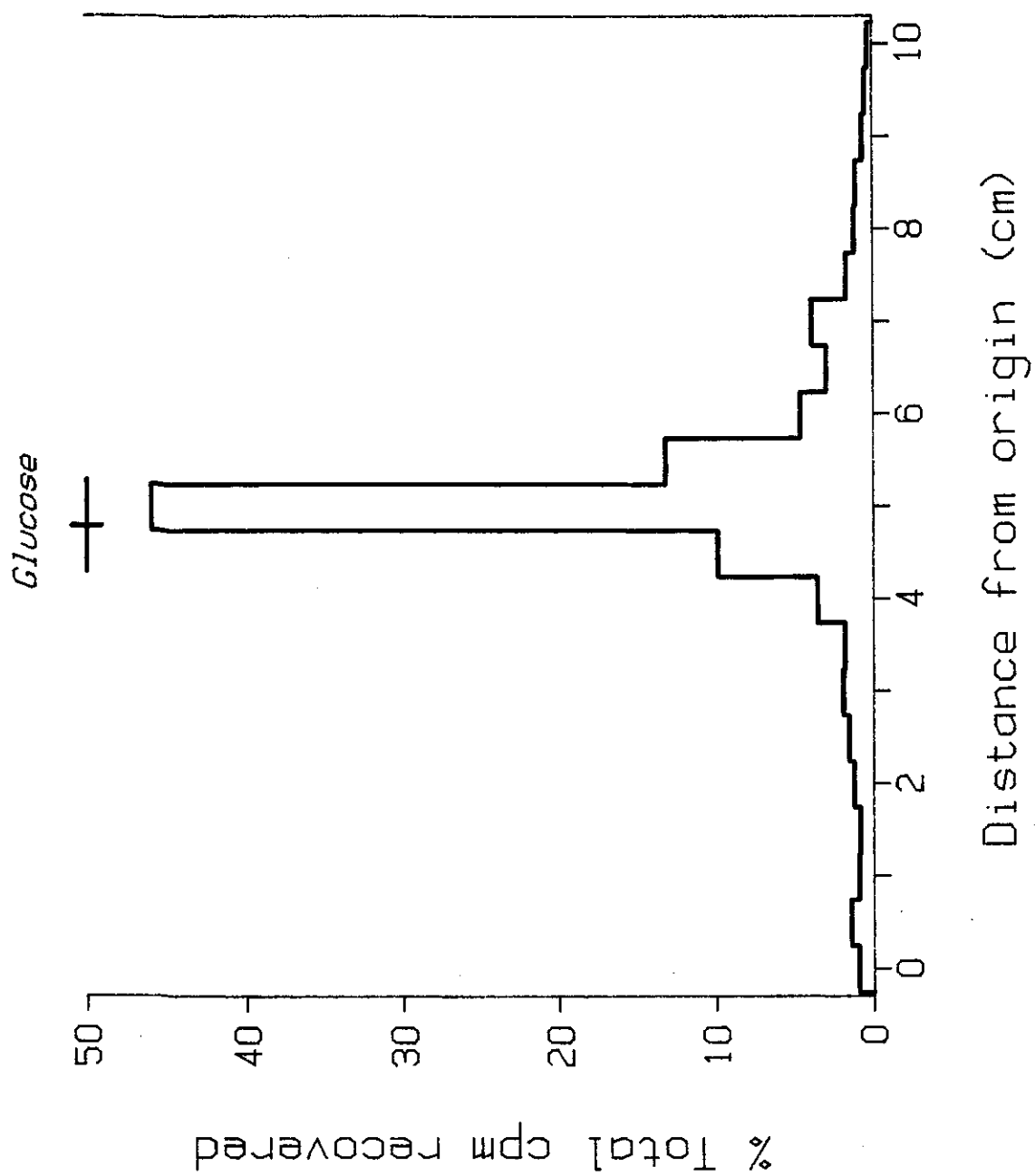
TABLE III-1

Transmural glucose fluxes across the isolated midgut of Callinectes sapidus

Mucosal [Glucose] (mM)	Transmural glucose fluxes (nmol cm ⁻² h ⁻¹)		
	J _{ms}	J _{sm}	J _{net}
Independent Determinations			
5.0	55.7 ± 5.1 (9)	15.7 ± 2.7 (6)	40.0 ± 5.8
Simultaneous Determinations			
5.0	62.7 ± 11.2 (3)	18.1 ± 4.2 (3)	44.6 ± 9.6 (3)
2.5	58.5 ± 8.9 (3)	32.6 ± 10.2 (3)	25.9 ± 5.7 (3)

Values are means ± SEM (no. of experiments). In independent experiments, unidirectional fluxes from mucosal to serosal (J_{ms}) and from serosal to mucosal (J_{sm}) were determined in separate pieces of tissues. In simultaneous experiments, they were determined by double labeling. In each case, glucose concentration in the mucosal medium was as indicated, while that of the serosal medium was maintained at 5 mM.

FIG. III-1. Radiochromatogram of serosal medium after unidirectional mucosal to serosal glucose flux experiments. Results are the mean of duplicate experiments. The vertical line on the top of the figure indicates the R_f of glucose standard (0.48) and the horizontal line the range observed.



tritiated activity resulted when compared to unprocessed samples. This finding indicates that part of the tritiated activity in the serosal medium is in the form of volatile tritiated water as a result of metabolism. However, it was noted that the drying procedure also led to a decrease of about 10% in the activity of the parent perfusing solution. Thus, the reduction in activity in the serosal samples might partially be due to an artifact, which was confirmed in experiments using the non-metabolizable 2- $^3\text{H}(\text{G})$ -deoxy-D-glucose (New England Nuclear) as the tracer where a decrease of tritiated activity was also evident after drying. Thus, the actual reduction of tritiated activity due to loss of metabolic water was less than 10 to 20%. Despite of this artifact, the chromatographic analysis of serosal medium indicates that at least 70% of tritiated activity translocated through the midgut is in the form of free glucose.

Transmural glucose fluxes as a function of glucose concentration

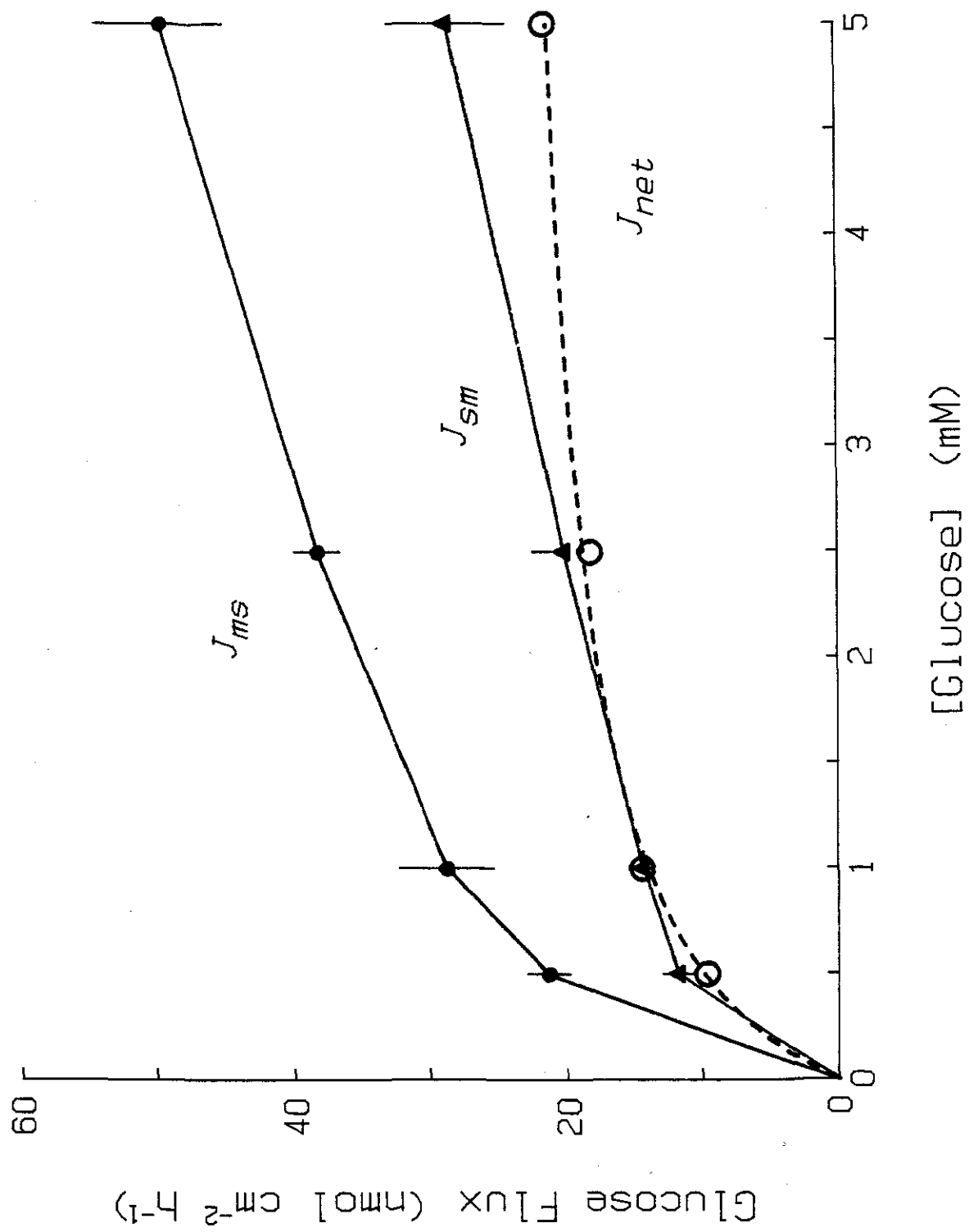
The fluxes J_{ms} and J_{sm} were hyperbolic functions of mucosal and serosal glucose concentrations, respectively (Fig. III-2). J_{ms} significantly exceeded J_{sm} throughout the range of glucose concentration tested (0.5 to 5.0 mM).

The difference between J_{ms} and J_{sm} gave the net flux, J_{net} , which followed Michaelis-Menten kinetics:

$$J_{net} = \frac{J_{max}[G]}{K_m + [G]}$$

where $[G]$ is the glucose concentration, J_{max} is the flux at saturation,

FIG. III-2. Variation of transmural glucose fluxes across the isolated midgut of Callinectes sapidus with glucose concentration. Data points of unidirectional fluxes from mucosal to serosal, J_{ms} (closed circles), and from serosal to mucosal, J_{sm} (closed triangles), are the mean of 4 experiments, and the bars represent \pm SEM. In some cases, half of the bar is omitted for clarity. The difference between the two fluxes gives the net flux, J_{net} (open circles). The dashed line representing J_{net} was computed from the Michaelis-Menten equation using the kinetic constants derived from the single reciprocal plot illustrated in Fig. III-3.



and K_m the $[G]$ where $J_{net} = J_{max}/2$. A single reciprocal plot of J_{net} vs. $J_{net}/[G]$ gave a K_m of 0.73 mM and J_{max} of $24.1 \text{ nmol cm}^{-2} \text{ h}^{-1}$ (Fig. III-3). It should be noted that this maximal flux determined is lower than the net flux determined in 5.0 mM glucose shown in Table III-1. This discrepancy is probably due to variability among individual tissues.

Inhibition of unidirectional mucosal to serosal glucose flux

Unidirectional mucosal to serosal glucose flux across the isolated midgut decreased significantly upon the addition of potassium cyanide or sodium azide, both inhibitors of oxidative metabolism, to the serosal medium (Fig. III-4a). The flux was also depressed by iodoacetic acid, an inhibitor of the glycolytic pathway.

Removal of Na^+ from the luminal medium also inhibited mucosal to serosal flux (Fig. III-4b). Ouabain (0.1 mM), a specific inhibitor of the ubiquitous $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$, reduced J_{ms} when administered to the serosal but not mucosal medium.

In contrast to ouabain, phlorizin (0.01 mM), a potent inhibitor of glucose transport in many animal tissues, depressed J_{ms} when added to the mucosal medium (Fig. III-4c). At a higher concentration (0.1 mM), phlorizin administered to the serosal medium also reduced J_{ms} significantly. Yet the degree of inhibition was much less than that observed when the same concentration of phlorizin was added to the perfusing medium.

The effects of three hexoses on J_{ms} of glucose are shown in Fig. III-4d. In the presence of equimolar D-galactose in the luminal medium, the unidirectional flux of 5.0 mM glucose was reduced by 30%. The same amount of L-glucose and D-fructose had no apparent effect on J_{ms} .

FIG. III-3. Single reciprocal plot of net transmural glucose flux, J_{net} vs. $J_{net}/[\text{Glucose}]$. K_m was determined from the slope and J_{max} from the vertical intercept of the regression line ($r^2 = 0.98$).

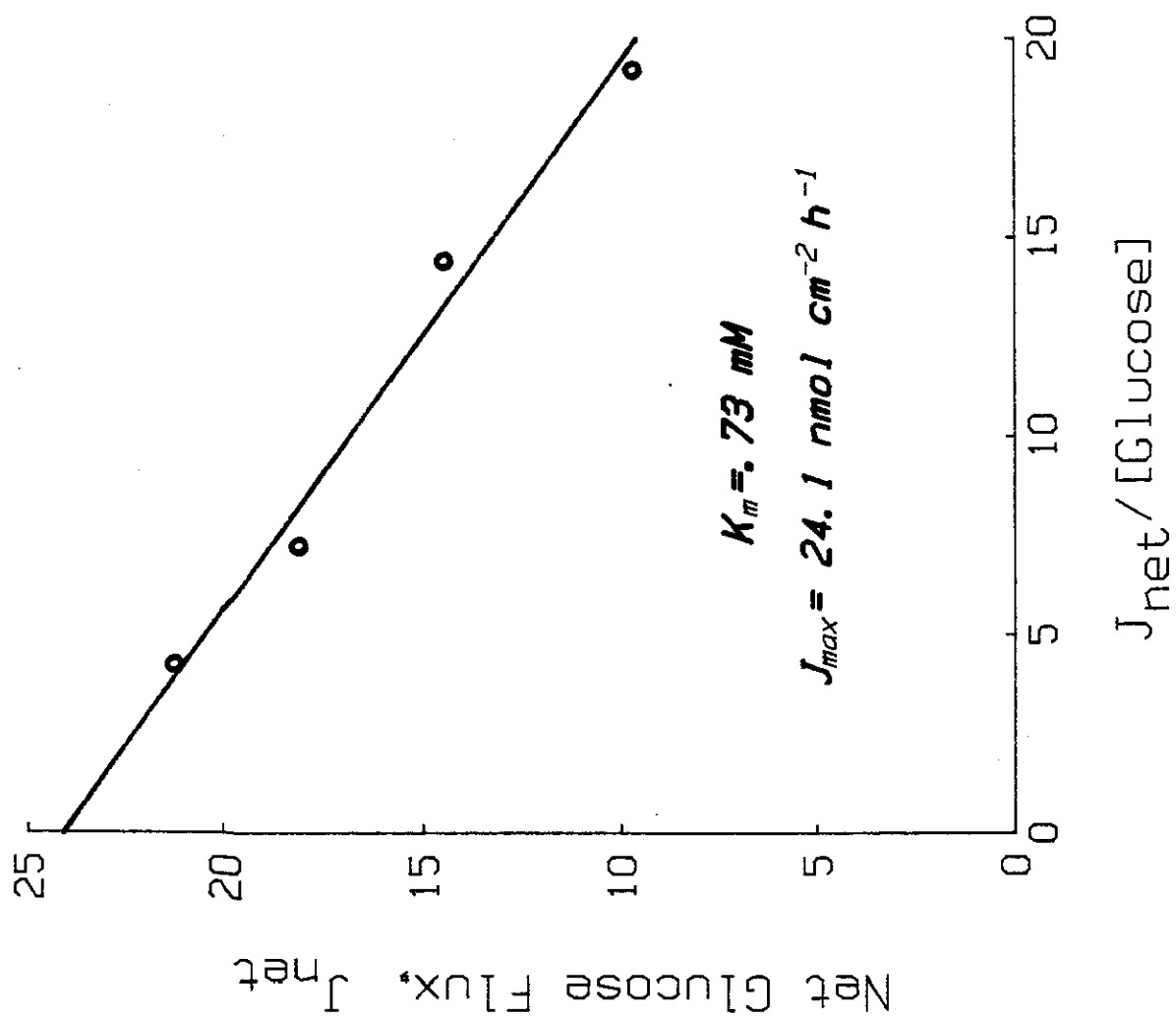


FIG. III-4a. Effects of the metabolic inhibitors, potassium cyanide, sodium azide, and iodoacetic acid, on unidirectional mucosal to serosal glucose flux across the isolated midgut of Callinectes sapidus. Results are expressed as mean % of initial flux (i.e., before treatment), and the vertical lines represent 1 SEM. Numbers of experiments are indicated in parentheses. Asterisks indicate significant differences from the control glucose flux.

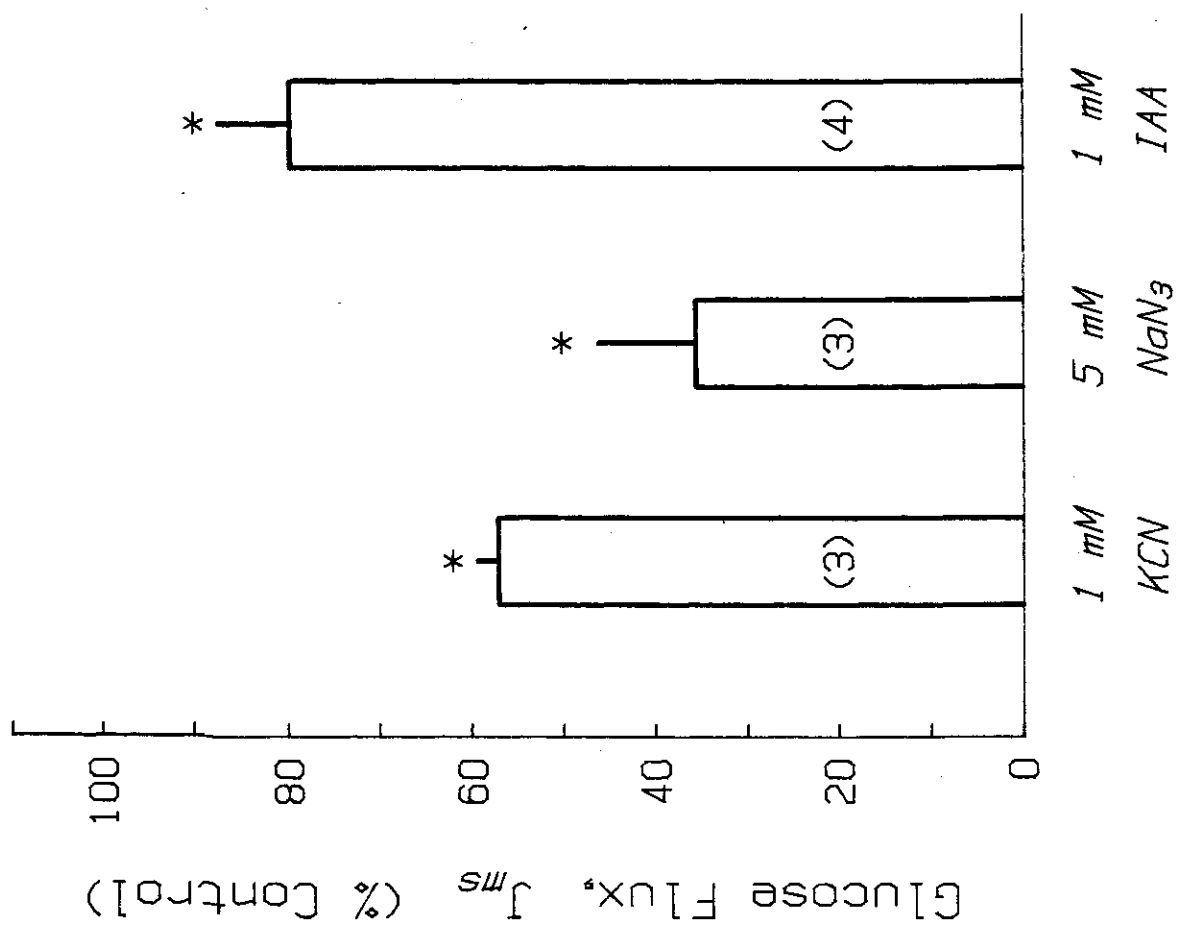


FIG. III-4b. Effects of Na^+ and ouabain on unidirectional mucosal to serosal glucose flux across the isolated midgut of Callinectes sapidus. The letters M or S within parenthesis indicate whether the treatment was administered on the mucosal or serosal medium, respectively. Refer to the legend of Fig. III-4a for further explanations.

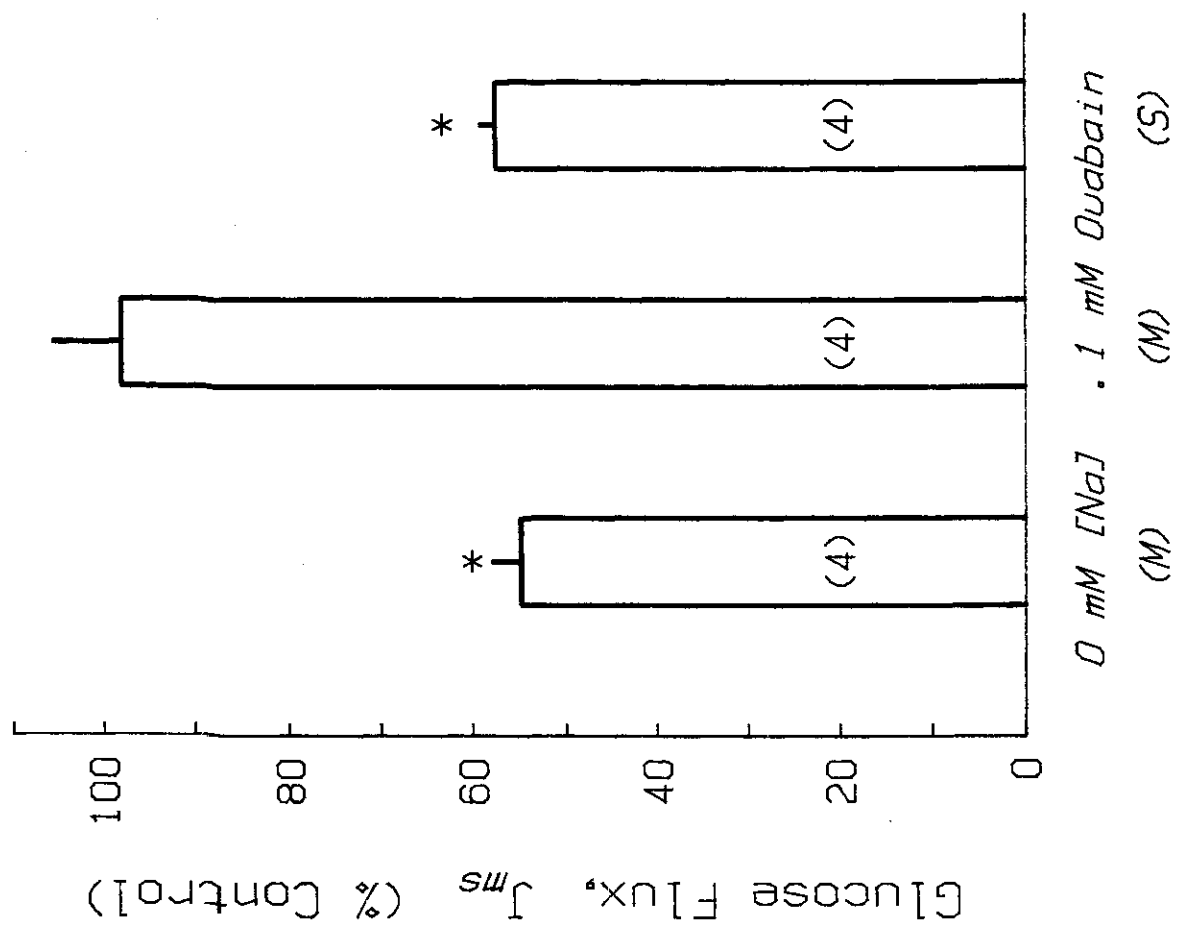


FIG. III-4c. Effect of phlorizin on unidirectional mucosal to serosal glucose flux across the isolated midgut of Callinectes sapidus.

Refer to legend of Fig. III-4a & b for explanations.

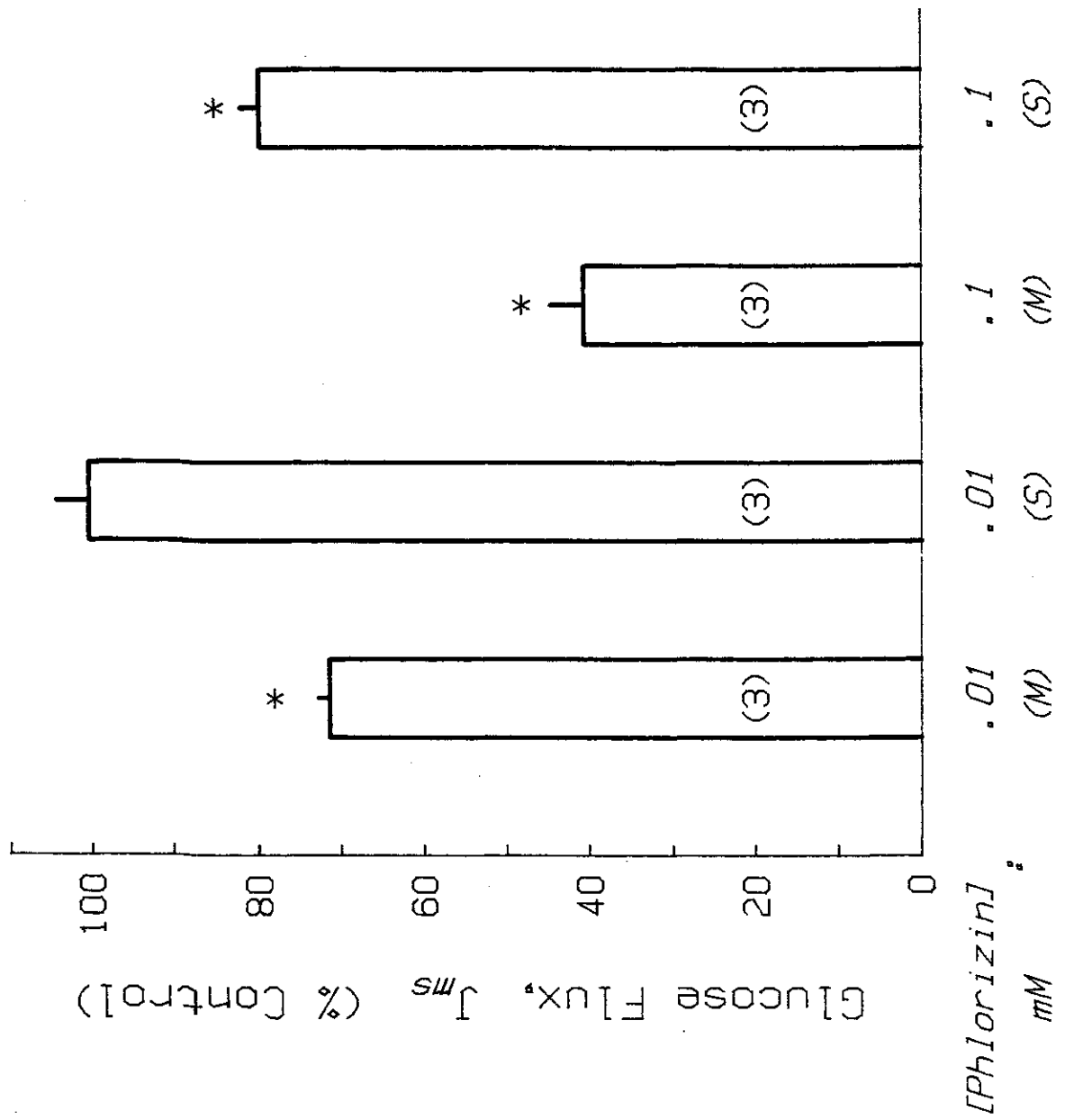
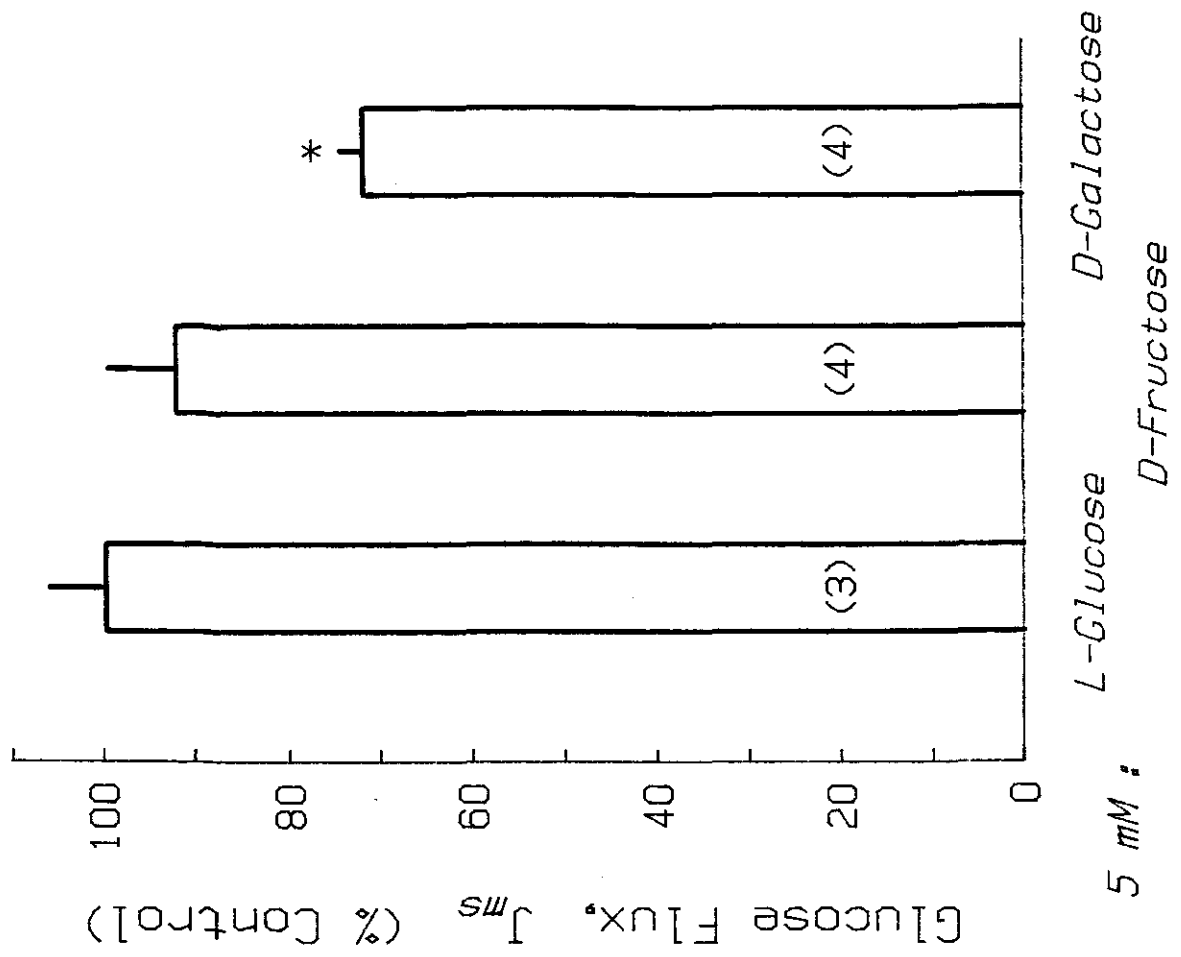


FIG. III-4d. Effects of the hexoses, L-glucose, D-fructose, and D-galactose, on unidirectional mucosal to serosal glucose flux across the isolated midgut of Callinectes sapidus. All hexoses were administered to the mucosal medium. Refer to the legend of Fig. III-4a for further explanations.



DISCUSSION

The present study illustrates that the isolated midgut of the blue crab, Callinectes sapidus, is capable of net glucose absorption, which is saturable as a function of external glucose concentration. This glucose transport process is Na^+ -dependent, a property in common with most active nutrient transport mechanisms across epithelia. Most works in this area tend to support the Na^+ -gradient hypothesis proposed by Crane (1965). According to this hypothesis, entry of nutrients into the cells is coupled to downhill movement of Na^+ . The low intracellular Na^+ concentration is maintained by active extrusion of Na^+ by $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ on the serosal border of the epithelium. The findings of the present study are compatible with this model. Ouabain, through its inhibitory effect on $(\text{Na}^+-\text{K}^+)\text{-ATPase}$, would most likely increase the intracellular Na^+ concentration. Given a smaller Na^+ gradient, mucosal entry of glucose is reduced, and so is the unidirectional mucosal to serosal flux. Inhibition of metabolism with potassium cyanide, sodium azide, and iodoacetic acid depresses glucose transport, probably due in large part to lack of ATP for phosphorylation of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$.

The unidirectional mucosal to serosal glucose flux across the midgut of Callinectes sapidus is also sensitive to phlorizin, a characteristic of the active glucose transport processes well established in mammalian intestine and kidney tubule (Lotspeich, 1960). Phlorizin is most sensitive when applied to the mucosal medium, suggesting that the

midgut of C. *sapidus*, like the above mammalian tissues, possesses a phlorizin-sensitive Na^+ -glucose cotransporter at its apical membrane. The reduction of glucose transport by 0.1 mM phlorizin administered to the serosal bath may be an indirect consequence of the inhibitory effect of a high concentration of phlorizin on the activity of various enzymes (see Crane, 1960 for a review), including the (Na^+-K^+) -ATPase (Britten & Blank, 1969; Robinson, 1969). The possibility of the presence of a moderately phlorizin-sensitive glucose transport system in the serosal border cannot be excluded.

Unidirectional mucosal to serosal glucose flux across the midgut of Callinectes *sapidus* was decreased by the presence of D-galactose, but not D-fructose and L-glucose. Na^+ -dependent mucosal glucose uptake inhibited by galactose was observed in the midgut of the freshwater shrimp, Macrobrachium *rosenbergii* (Ahearn & Maginniss, 1977). This inhibitory effect may be due to the interaction of galactose with the glucose binding site, implicating that the two hexoses share a common transport system. A common transport mechanism for D-glucose and galactose are found in the intestine and proximal tubule in the kidney of mammals (see Crane, 1960; Kinne, 1976 for reviews). However, since the nature of competition of galactose with glucose transport was not investigated in the midgut of C. *sapidus* nor M. *rosenbergii*, the notion of a common transport mechanism for the two hexoses in the crustacean gut have not been verified. Other mechanisms, such as sharing a limited energy resource for uptake between the two hexoses or a change of transmembrane potential induced by galactose, can also lead to a decrease in mucosal to serosal glucose flux.

This study also demonstrates that glucose absorption across the isolated midgut of Callinectes sapidus can take place against a concentration gradient. Further, the transport mechanism has a relatively high affinity for glucose. These characteristics may be physiologically advantageous to the animal. It is widely believed that the hepatopancreas of crustaceans is the major site of nutrient absorption (see Gibson & Barker, 1979; Dall & Moriarty, 1983 for recent reviews). Presumably, nutrient levels in the lumen of the tubular midgut would be low. Under these conditions, a high-affinity glucose transport system capable of uphill transport is desirable for more complete uptake of the sugar. A similar salvage function of the midgut in nutrient absorption has also been suggested in M. rosenbergii (Ahearn & Maginniss, 1977).

The magnitude of the net glucose absorption across the midgut of Macrobrachium rosenbergii is $103.9 \pm 23.1 \text{ nmol cm}^{-2} \text{ h}^{-1}$ (1 mM glucose; Wyban, Ahearn & Maginniss, 1980). Noting that this net flux is lower than nutrient fluxes determined in mammalian intestine, Ahearn (1982) asserted that the crustacean midgut delivers less quantity of nutrients to the blood per unit surface area than does mammalian intestine, and accordingly, the midgut plays a minor role in total nutrient absorption. Additional evidence is provided by mucosal uptake studies demonstrating low maximal influx rates and high affinity for nutrients (Ahearn, 1974, 1976; Ahearn & Maginniss, 1977; Brick & Ahearn, 1978). These results also provide indirect evidence for the importance of the hepatopancreas in nutrient uptake. The net glucose flux determined in the midgut of

Callinectes sapidus ($40.0 \pm 5.8 \text{ nmol cm}^{-2} \text{ h}^{-1}$; 5 mM glucose) is about half of that in M. rosenbergii. Thus, this finding supports the above suggestion. It should be noted, however, that comparison between transport data from studies of in vitro preparations using different experimental techniques can be misleading (see Chapter IV).

In conclusion, the results of the present study are consistent with the hypothesis that transmural glucose transport across the midgut of Callinectes sapidus is mediated by a luminal Na^+ -dependent, phlorizin-sensitive transport mechanism, the operation of which is dependent on the functioning of the basolateral $(\text{Na}^+-\text{K}^+)\text{-ATPase}$. Thus, the glucose transport system in the midgut of this crustacean species exhibits many basic properties characteristic of nutrient transport processes of the gastrointestinal tract of mammals as well as other animals, suggesting that this type of active nutrient transport mechanism is widely distributed in the animal kingdom.

CHAPTER IV

GLUCOSE TRANSPORT BY THE IN VITRO PERFUSED MIDGUT

OF CALLINECTES SAPIDUS

B. MUCOSAL UPTAKE

INTRODUCTION

There are numerous studies dealing with the digestive functioning of the crustacean gut (see Dall & Moriarty, 1983 for a review), but relatively little work has been done on the nutrient absorptive process. Recent studies on transport processes in the crustacean gut have been reviewed by Ahearn (1982). Most investigations on the mechanisms of nutrient uptake have been directed at the tubular midgut (Ahearn, 1974, 1976; Ahearn & Maginniss, 1977; Brick & Ahearn, 1978). These studies showed that whereas the tissue has many transport properties in common with the mammalian intestine, its role in nutrient absorption may be limited. This notion has been supported by my previous study on transmural glucose absorption across the isolated midgut of the blue crab, Callinectes sapidus (Chapter III). I have shown that most glucose translocated across the midgut is in the form of free glucose, and glucose transport in this tissue is energy- and Na^+ -dependent and exhibits saturation kinetics. Moreover, the presence of a Na^+ -dependent phlorizin-sensitive mucosal uptake mechanism was suggested. The present investigation was performed to clarify the mucosal glucose uptake step in the same preparation. Kinetic studies confirm the presence of a Na^+ -dependent phlorizin-sensitive saturable mechanism for glucose transport across the mucosal border. A Na^+ -independent, apparently diffusive pathway, is also involved. Further, glucose entering the midgut appears predominantly as phosphorylated forms, suggesting that efflux of free glucose across the serosal border requires an active mechanism.

MATERIALS AND METHODS

Procedures for the maintenance of animals and the composition of physiological salines used in this study have been described (Chapter II).

Determination of mucosal glucose uptake

The in vitro perfusion technique described in Chapter II was adapted to determine mucosal uptake of glucose by the midgut. After mounting the midgut in the perfusion chamber, it was perfused with glucose-free saline for 3 min and then with saline containing glucose together with [6-³H]-D-glucose as the tracer of glucose uptake and [1,2-¹⁴C]-polyethylene glycol (M.W. 4000) as a marker for extracellular space. The length of perfusion time varied from 1.5 to 60 min. At the end of the perfusion period, the serosal medium was immediately replaced with ice-cold saline and the gut was flushed with the same solution. The mounted midgut was then cut between the catheters, blotted gently, weighed, and placed in a scintillation vial. The tissue was moistened with 0.1 ml distilled water and solublized in 0.5 ml Protosol at 40°C overnight. After cooling, 3.4 ml of distilled water and 10 ml of Aquasol were added. Radioactivity was determined with a Beckman LS-100C liquid scintillation counter. Activity of perfusing saline and tissue samples was corrected for quenching with automated external standardization to obtain disintegrations per min. After corrections for the spillover of ¹⁴C activity to the ³H window and extracellular tritiated activity, the amount of glucose uptake was computed. Radiochemicals, Protosol and Aquasol were obtained from New England Nuclear.

The effect of luminal Na^+ on mucosal glucose uptake was investigated by perfusing the midgut with radioactively labeled sodium-free saline. The effects of various inhibitors were studied by incubating the midgut in saline containing the inhibitor for 15 min before mucosal uptake determination.

To express fluxes, two measurements of the quantity of tissue were used, wet weight to the nearest 0.01 mg and surface area estimated by considering the gut as a cylinder and expressed in cm^2 . The two were linearly correlated (Fig. IV-1). In this report, uptake was expressed as nmol of glucose per cm^2 of tissue per min. The other measurement essentially gives the same results.

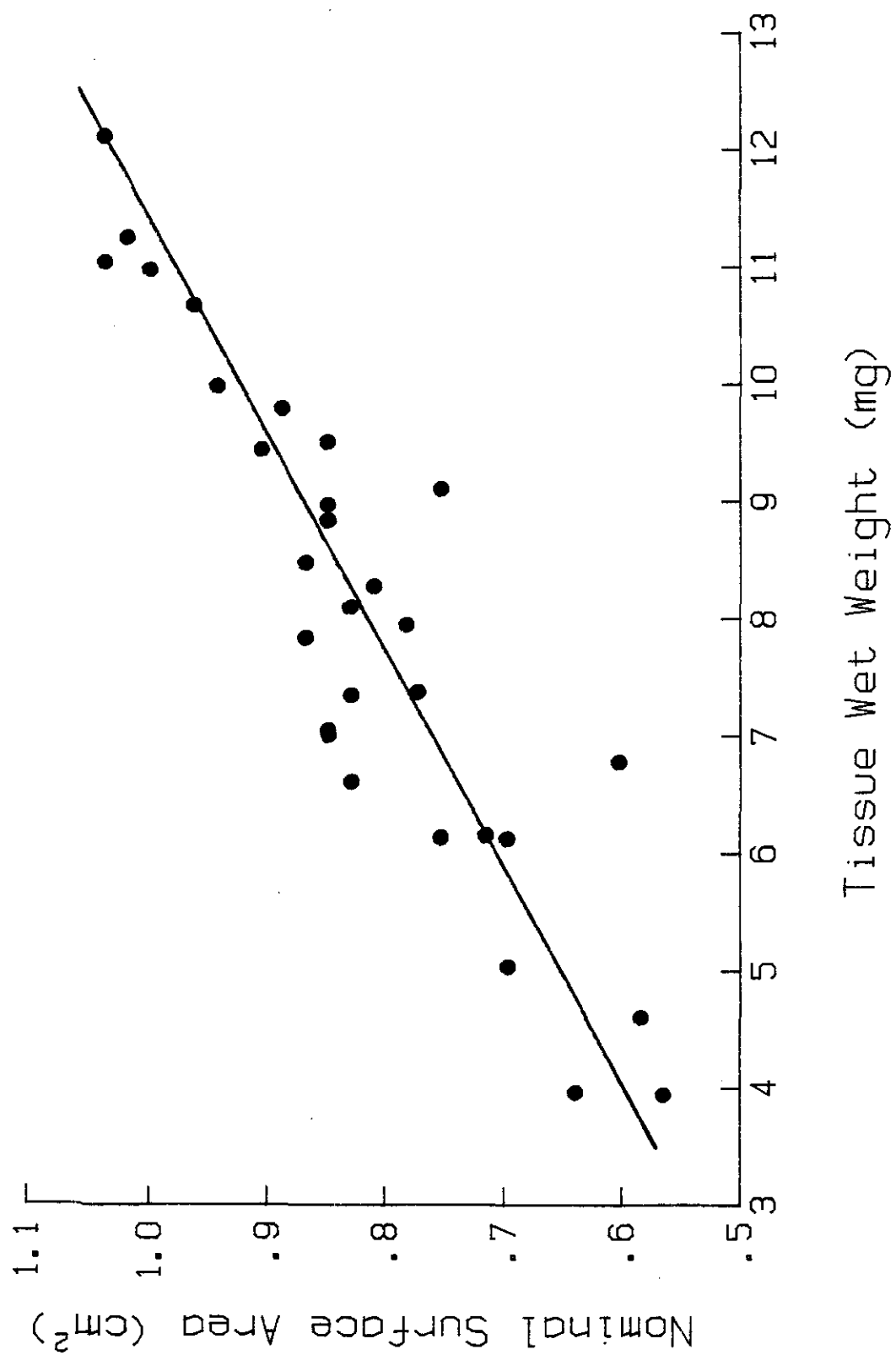
Thin layer chromatography and enzyme treatment

The chemical state of tritiated glucose absorbed by the midgut was investigated with thin layer chromatography. The tissues after uptake experiments were extracted in 2 ml of 70% ethanol for 24 h. Preliminary experiments showed that more than 95% of total radioactivity was extracted within this period of time. The chromatographic procedure is described in Chapter III. In addition to labeled and unlabeled glucose standards, the R_f of glucose-6-phosphate standard (Sigma) was also determined.

The presence of phosphorylated glucose compounds was further investigated in a few experiments described as follows. Ethanol extract from a single tissue was divided into two portions for analysis. Both were evaporated to dryness and redissolved in an equal volume of pH 9

Fig. IV-1. Relation between the nominal surface area and wet weight of the isolated midgut of Callinectes sapidus. The surface area was estimated by considering the midgut as a cylinder. The wet weight was determined to the nearest 0.01 mg with a micro-balance. The linear regression line ($r^2 = 0.83$; $n = 30$) is:

$$\text{Surface area (cm}^2\text{)} = 0.0532 \times \text{Wet weight (mg)} + 0.39$$



Tris buffer. One sample was treated with alkaline phosphatase (Sigma) for 60 min at 37°C. Another portion, acting as control, was processed identically except that the phosphatase was denatured by heat treatment at 100°C for 60 min beforehand. The two samples were then evaporated to dryness again, redissolved in 70 % ethanol, and analysed by chromatographic procedure as before.

Statistical analysis

Mean values of flux were compared using Student's t-test. If the variance ratio indicated unequal variances, a modified t-test was used instead (Baily, 1959). Significant difference between mean values was assumed at a probability level of 0.05.

RESULTS

Effect of perfusion rate on mucosal glucose uptake

Mucosal glucose uptake of 1 mM glucose by the in vitro perfused midgut of Callinectes sapidus was measured using three different perfusion rates, 50, 100, and 150 $\mu\text{l min}^{-1}$ and an exposure period of 3 min (Fig. IV-2). There are no significant differences among the three mean values of mucosal uptake. However, a general trend of increased uptake with perfused rate was suggested. This increase can be explained by a decrease in the thickness of the unstirred layer outside the mucosal border with increasing perfusion rate, thus lowering the resistance of the barrier to glucose uptake. This increase in uptake with perfusion rate might also be due to an elevation in hydrostatic pressure so that the gut was distended exposing a larger surface area for influx of glucose. All experiments reported thereafter were done at a perfusion rate of 100 $\mu\text{l min}^{-1}$.

Mucosal glucose uptake as a function of time

The uptake of 1 mM glucose by the midgut between 1.5 to 60 min is shown in Fig. IV-3. The midgut accumulated glucose rapidly with time and the uptake gradually leveled off. During this time period, the apparent extracellular space (expressed as % of tissue wet weight) estimated by the uptake of [^{14}C]-polyethylene glycol also increased (Fig. IV-4). The initial rapid increase apparently represents entry of the marker into the extracellular space. The slow increase after this initial period is

FIG. IV-2. Effect of perfusion rate on mucosal glucose uptake by the isolated midgut of Callinectes sapidus. Data points are the mean from 4 or 5 tissues, and bars represent \pm SEM.

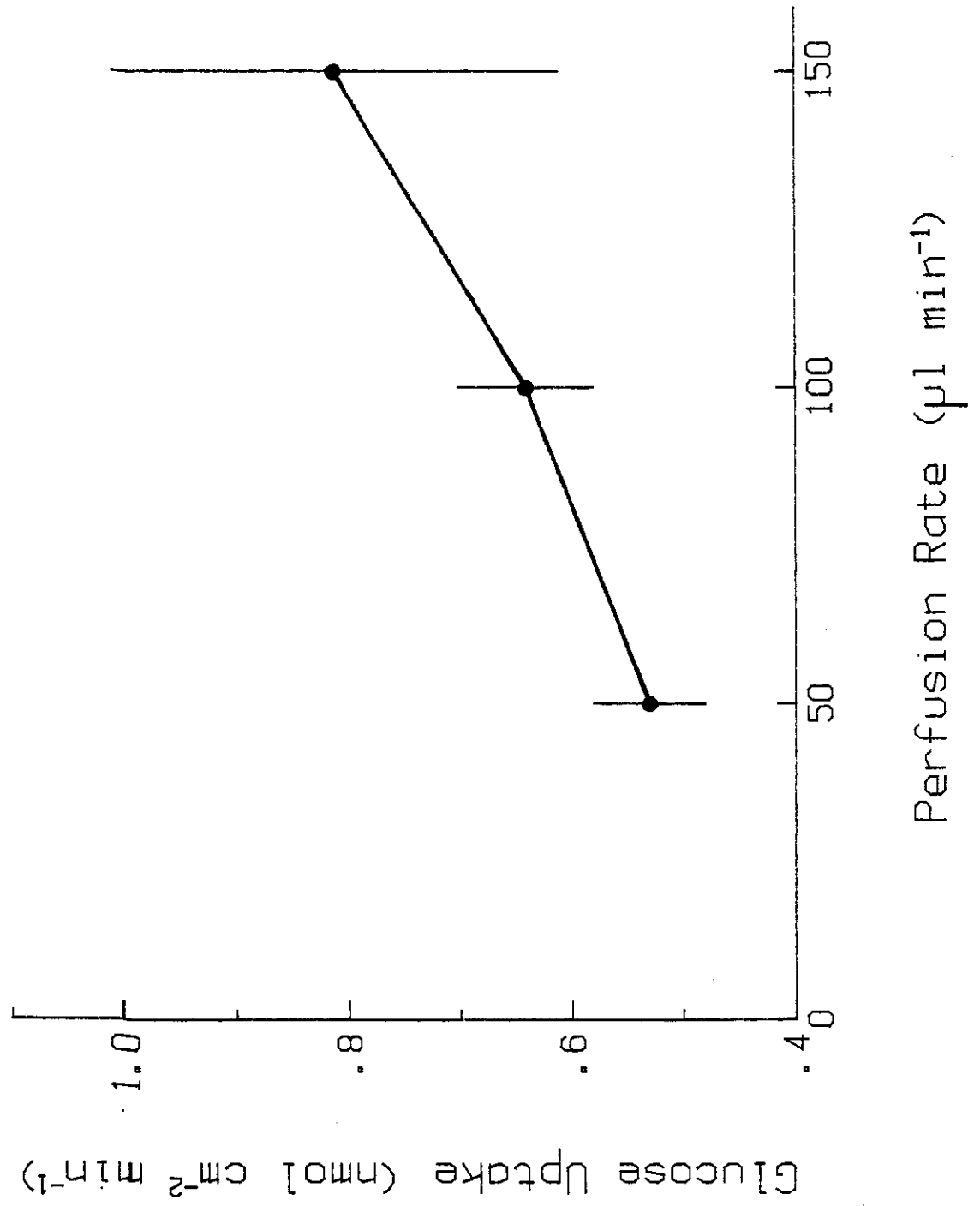


FIG. IV-3. Time course of mucosal glucose uptake (1 mM glucose) by the isolated midgut of Callinectes sapidus in the presense (460 mM; closed circles) and absence (closed triangles) of Na^+ . Data points are the mean of 3-6 experiments, and the bars represent \pm SEM. If no bars are shown, they are within the region of the symbol.

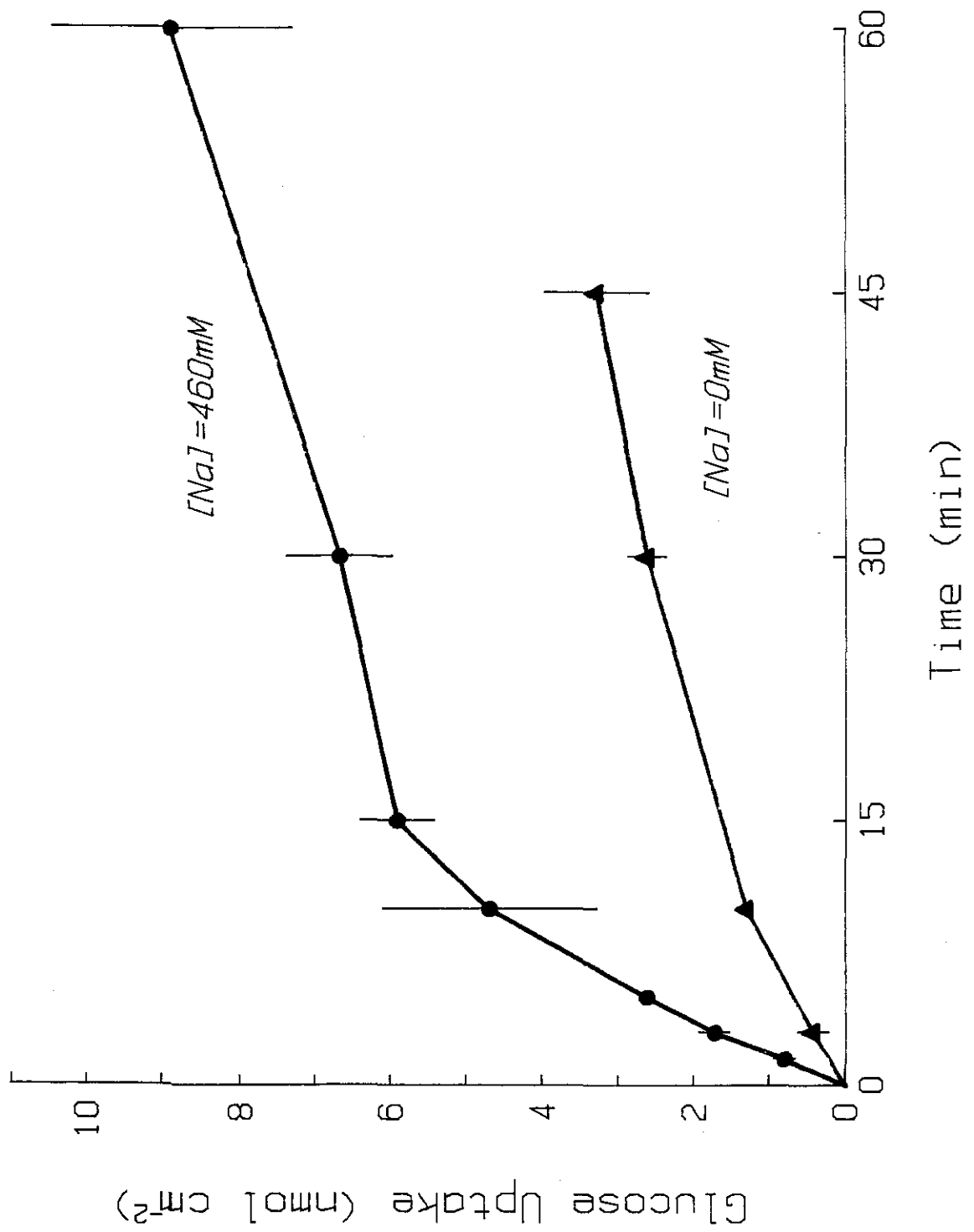
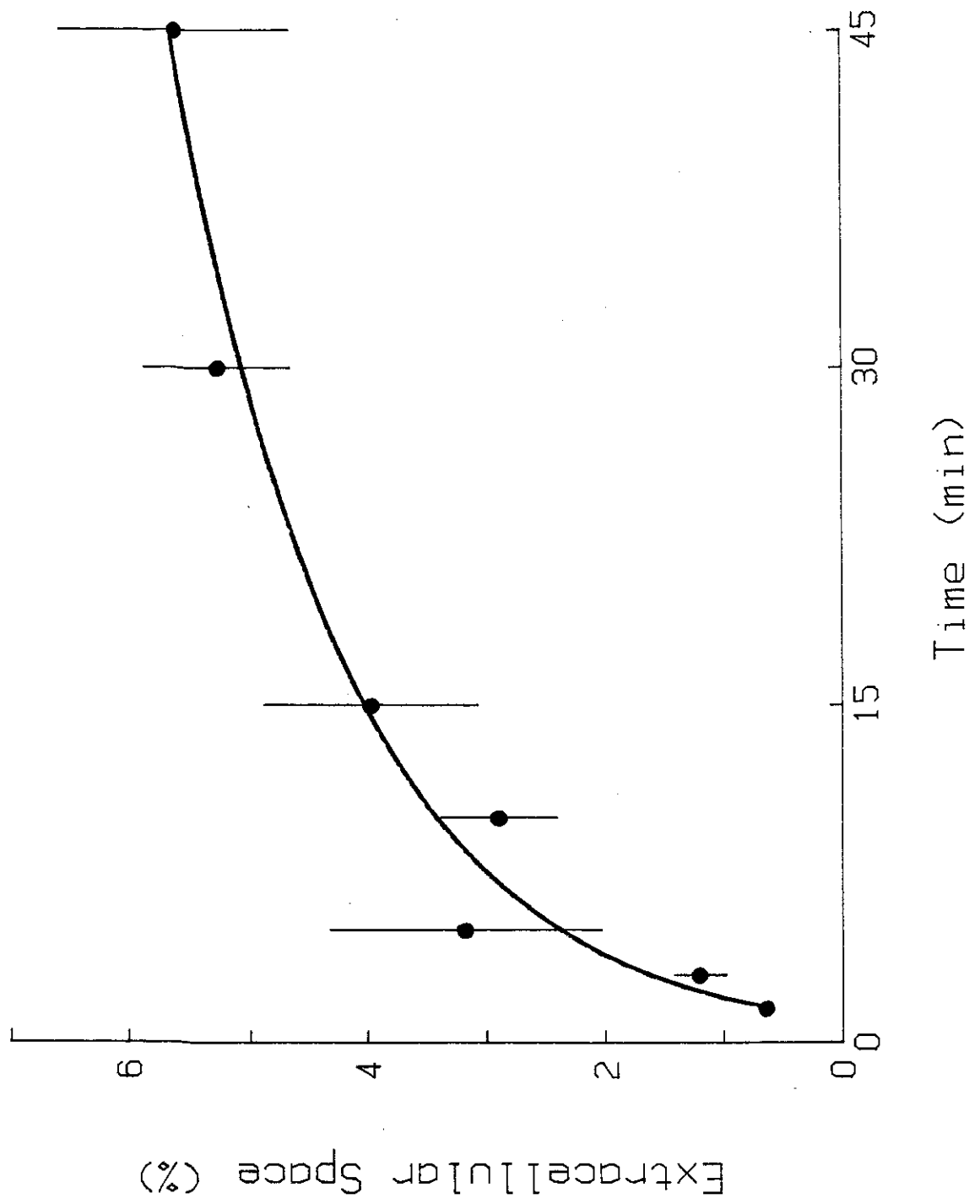


FIG. IV-4. Extracellular space of the isolated midgut of Callinectes
sapidus as a function of time. Data points are the mean of 3-7
experiments, and the bars represent \pm SEM. If no bars are
shown, they are with the region of the symbol.



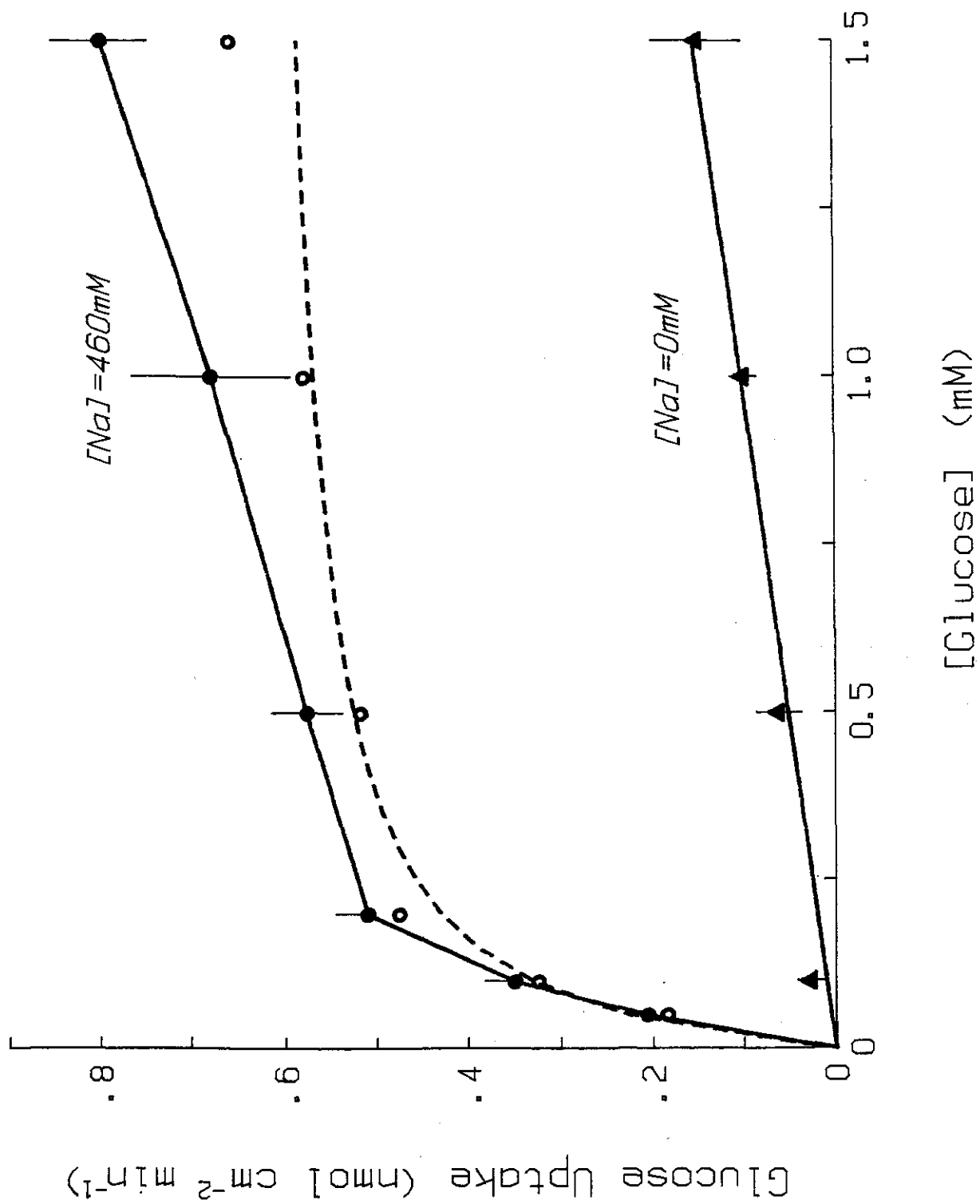
probably due to the gradual penetration of the marker into the cells through diffusion and mass transfer processes. Even in prolonged perfusion periods, the correction for apparent extracellular space represents only a small fraction of less than 10% of total glucose uptake. With sodium-free saline as the perfusate, the uptake was markedly reduced (Fig. IV-3). After 30 min of exposure, the glucose uptake in the absence of Na^+ made up less than 40% of the total uptake.

The glucose uptake measured in these experiments represents the sum of several processes including mucosal influx, metabolism, fluxes on the serosal border, as well as backflux into the lumen. During prolonged perfusion, these processes apparently approached steady state as indicated by the near plateau for glucose uptake. In order to minimize the contribution of processes other than mucosal influx, an appropriate perfusion time should be selected within the linear phase of the time course of mucosal uptake. In the presence or absence of Na^+ , the uptake was linear for the first ten minutes. Over this perfusion period, the intercepts of the two linear regression lines are not significantly different from zero. Thus, a standard incubation time of 3 min was chosen to determine initial rate of uptake in subsequent experiments.

Glucose uptake as a function of glucose concentration

To investigate the kinetics of glucose uptake, the midgut was perfused with salines containing 0.05 to 1.5 mM glucose. Mucosal uptake showed a hyperbolic relationship to glucose concentration (Fig. IV-5). With the removal of Na^+ in the luminal medium, glucose uptake was essentially a linear function of external glucose concentration, with a

FIG. IV-5. Mucosal glucose uptake as a function of glucose concentration in the presence (460 mM; closed circles) and absence (closed triangles) of Na^+ . Data points are the mean of 4-6 experiments, and bars represent \pm SEM. In some cases, half of the bar is omitted for clarity. If no bars are shown, they are within the region of the symbol. The difference of the fluxes in the two conditions gives Na^+ -dependent glucose uptake (open circles). The dashed line representing this uptake was computed from the Michaelis-Menten equation using the kinetic constants derived from the single reciprocal plot illustrated in Fig. IV-6.



vertical intercept that is not significantly different from zero. This finding indicates that glucose influx in the absence of luminal Na^+ is through a diffusive pathway. The apparent diffusion constant, K_d , of this Na^+ -independent glucose entry given by the slope of the linear regression line has a value of $8.3 \times 10^{-2} \text{ nmol cm}^{-2} \text{ min}^{-1}/\text{mM}$ glucose. The product of this constant multiplied by the glucose concentration was subtracted from the total uptake at each concentration studied to give the Na^+ -dependent mucosal glucose uptake. The Na^+ -dependent uptake of glucose, J , followed saturation kinetics described by the Michaelis-Menten equation:

$$J = \frac{J_{\max} [G]}{K_m + [G]} \quad (1)$$

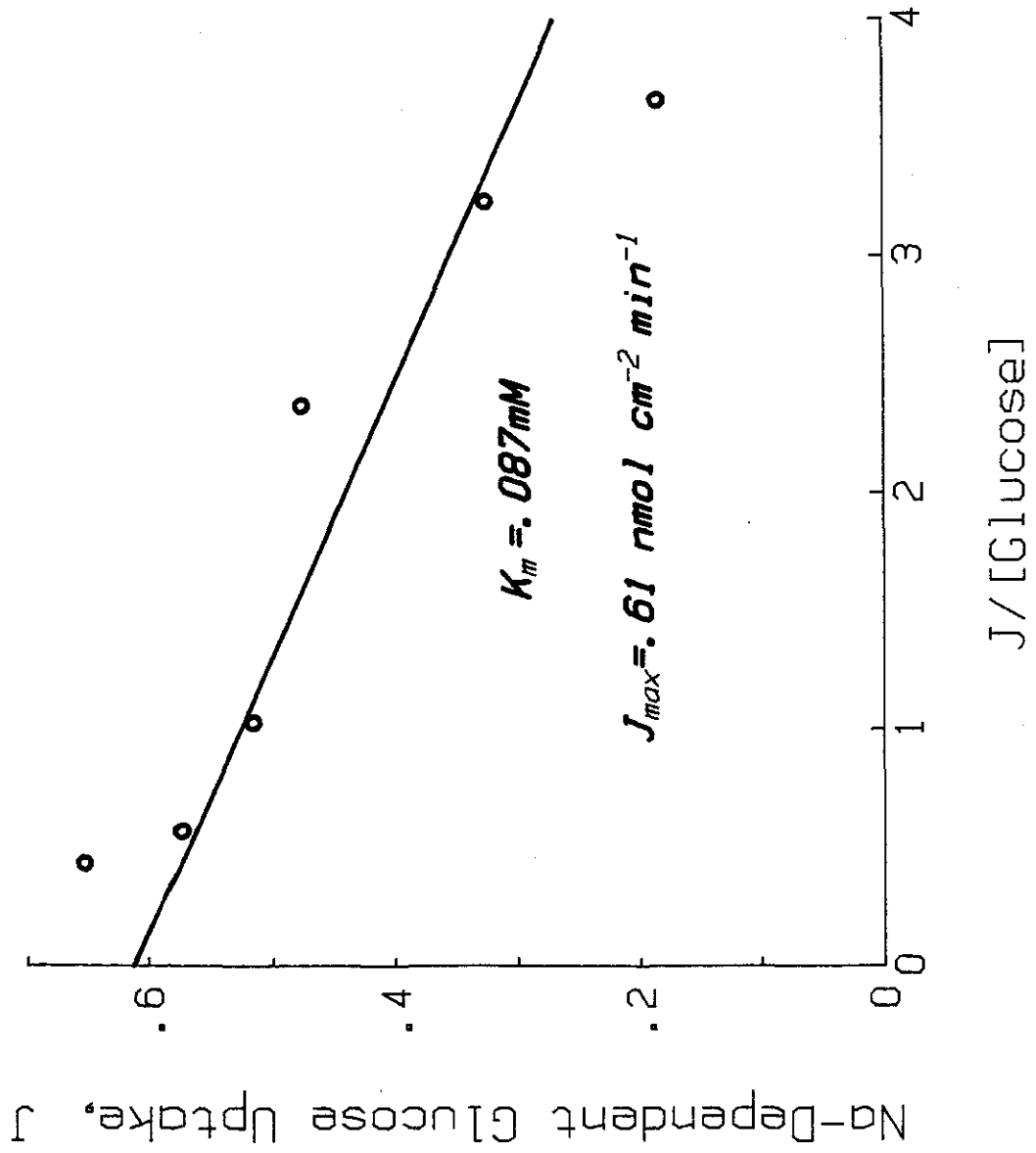
where $[G]$ is the luminal glucose concentration, J_{\max} is the maximal J , K_m is the half-saturation constant. The K_m and J_{\max} were computed by the single reciprocal plot of J vs. $J/[G]$ (Fig. IV-6). The corresponding values are $0.087 \pm 0.019 \text{ mM}$ and $0.61 \pm 0.04 \text{ nmol cm}^{-2} \text{ min}^{-1}$. These results indicate that Na^+ -dependent glucose uptake is a carrier-mediated process.

Thus, the total glucose uptake, J_{tot} , can be represented by the following relationship:

$$J_{\text{tot}} = \frac{J_{\max} [G]}{K_m + [G]} + K_d [G] \quad (2)$$

The first term is a Na^+ -dependent, saturable component and the second term a Na^+ -independent non-saturable counterpart.

FIG. IV-6. Single reciprocal plot of Na^+ -dependent glucose uptake, J vs. $J/[\text{Glucose}]$. Each point is the mean of 5-6 determinations at the same glucose concentration. The linear regression line was computed from all determinations ($n = 34$; $r^2 = 0.43$). K_m was derived from the slope, and J_{max} from the vertical intercept of the regression line.



Inhibition of mucosal glucose uptake

The effects of metabolic inhibitors on uptake of 1 mM glucose by the midgut are shown in Fig. IV-7. Both sodium azide and iodoacetic acid reduced glucose uptake, suggesting that it is dependent on energy resources derived from both aerobic and anaerobic pathways. As already shown, removal of Na^+ in the luminal solution significantly decreased glucose uptake. However, when the midgut was pre-incubated in saline with azide, the glucose uptake determined in sodium-free saline is not significantly different from influxes determined in either treatment. This result indicates that Na^+ -independent glucose uptake does not utilize energy derived from oxidative metabolism and thus provides additional evidence that this route of glucose transport is a diffusive pathway into the cells.

Uptake of glucose by the midgut was markedly reduced by the luminal presence of phlorizin (0.05 mM), a potent inhibitor of glucose uptake in many animal tissues. Ouabain, the specific inhibitor of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$, when added to the serosal medium, also inhibited glucose uptake, indicating that the functioning of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ is required for mucosal entry of glucose.

Fate of glucose after mucosal uptake

Thin layer chromatographic analysis of ethanol extracts from midgut exposed to 0.1 mM glucose for 3 min illustrates that less than 10% of the intracellular tritiated activity was in the form of glucose ($R_f = 0.48$), as shown in Fig. IV-8a. The major peak had a R_f indistinguishable from

FIG. IV-7. Effects of inhibitors and Na^+ on the mucosal glucose uptake by the isolated midgut of Callinectes sapidus. Results are expressed as mean values of (N) experiments, and vertical lines represent 1 SEM. Letters M or S within parenthesis indicate whether the treatment was administered on the mucosal or serosal medium, respectively.

Abbreviations: azide - sodium azide; IAA - iodoacetic acid

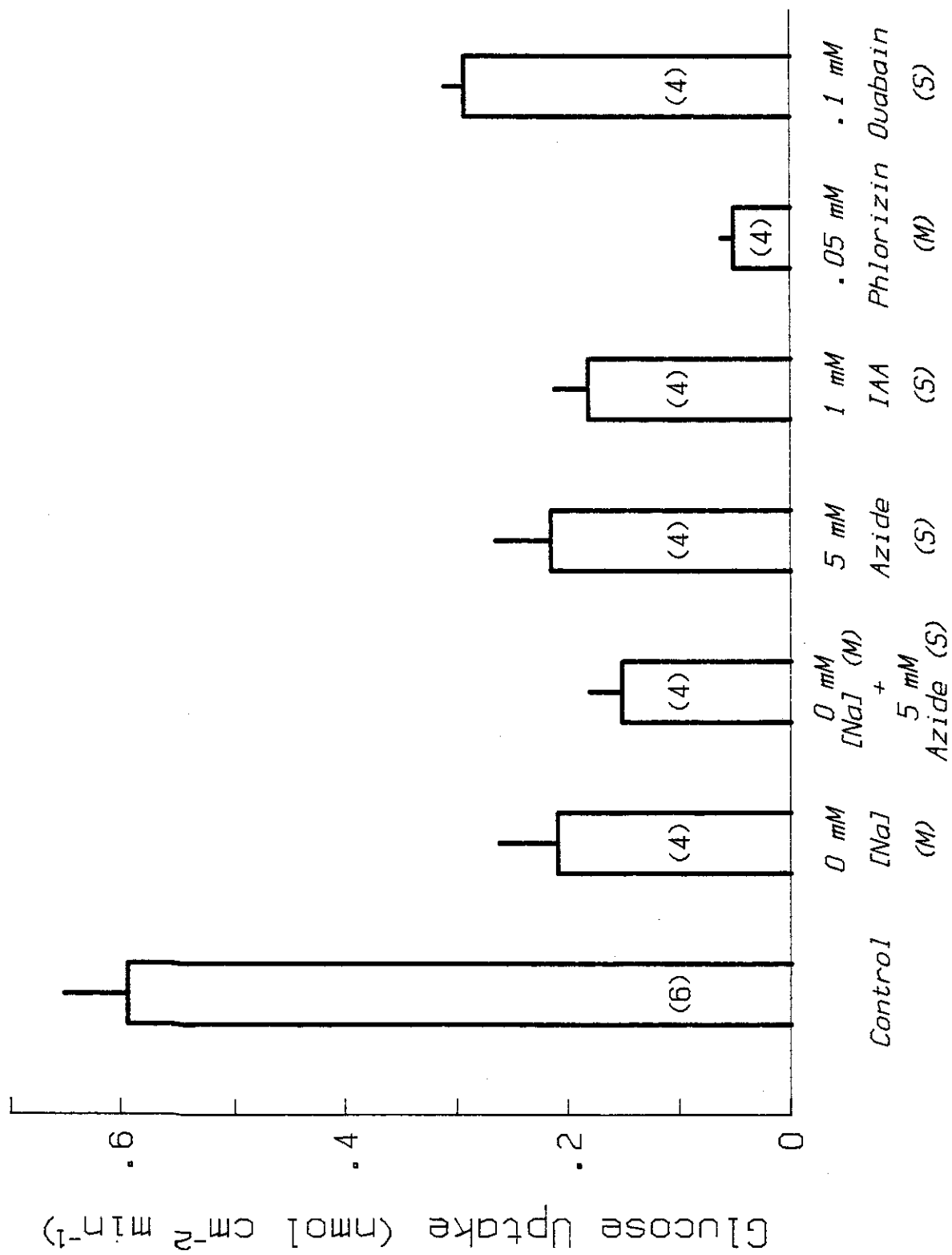
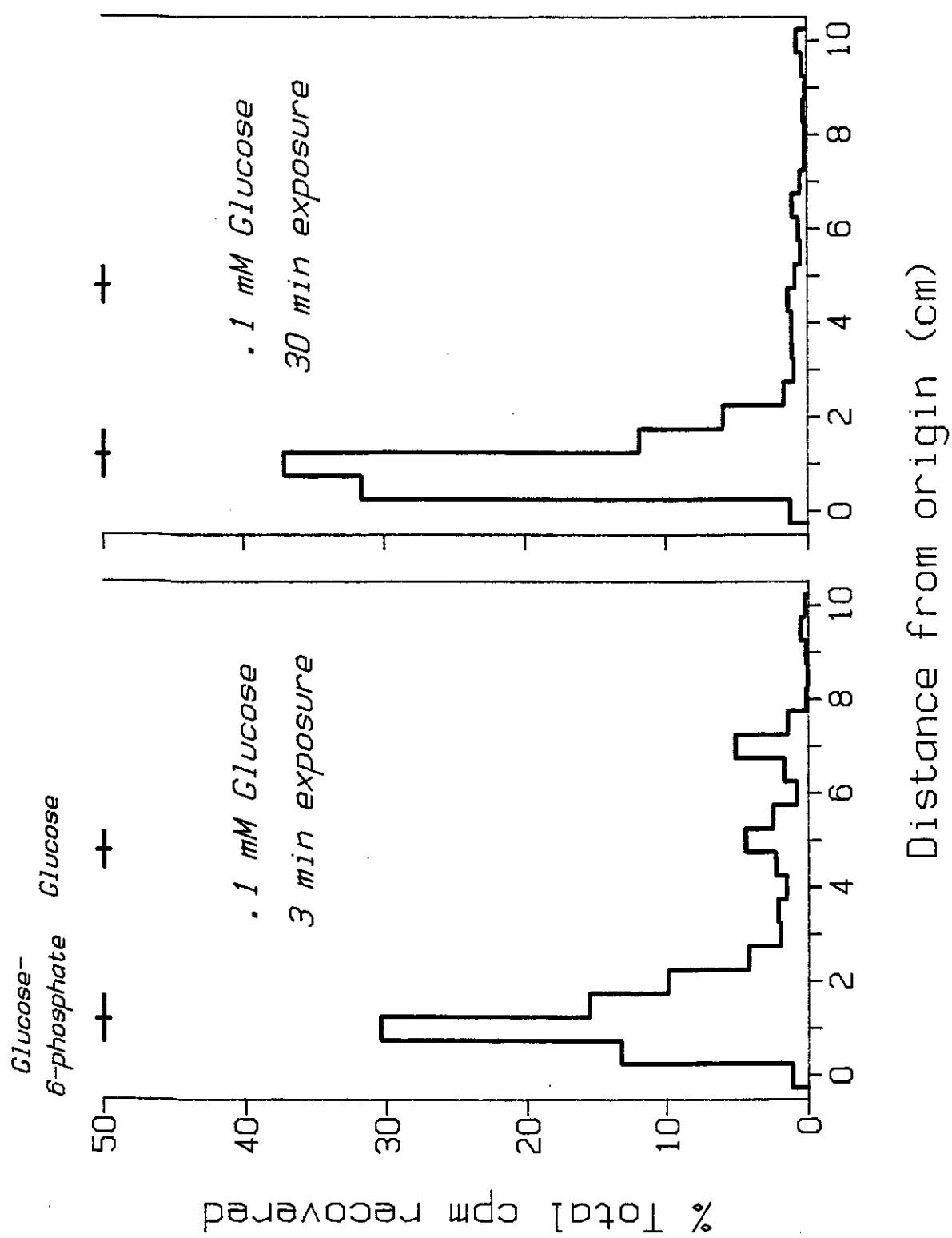


FIG. IV-8a. Radiochromatogram of ethanol extracts of midgut of Callinectes sapidus from mucosal uptake experiments using 0.1 mM glucose. Each chromatogram is the mean of duplicate experiments. At the top of the figure, the R_f and range of glucose ($R_f = 0.48$) standard are represented by vertical and horizontal lines respectively. The same parameters of glucose-6-phosphate ($R_f = 0.12$) are also shown.



that of glucose-6-phosphate ($R_f = 0.12$). An increase of either luminal glucose concentration to 1.0 mM and/or the exposure period to 30 min did not alter this distribution pattern of tritiated activity (Fig. IV-8a and b). These results suggest that most of the glucose absorbed in the midgut epithelium is probably in the phosphorylated form(s). Experiments with alkaline phosphatase provides additional evidence for this interpretation. As shown in Fig. IV-8c, treating the midgut extract with the enzyme before chromatographic analysis resulted in two peaks of tritiated activity, one coinciding with the peak exhibited by the control tissues and the other residing in the glucose region.

FIG. IV-8b. Radiochromatogram of ethanol extracts of midgut of Callinectes sapidus from mucosal uptake experiments using 1.0 mM glucose. See legend of Fig. IV-8a for explanations.

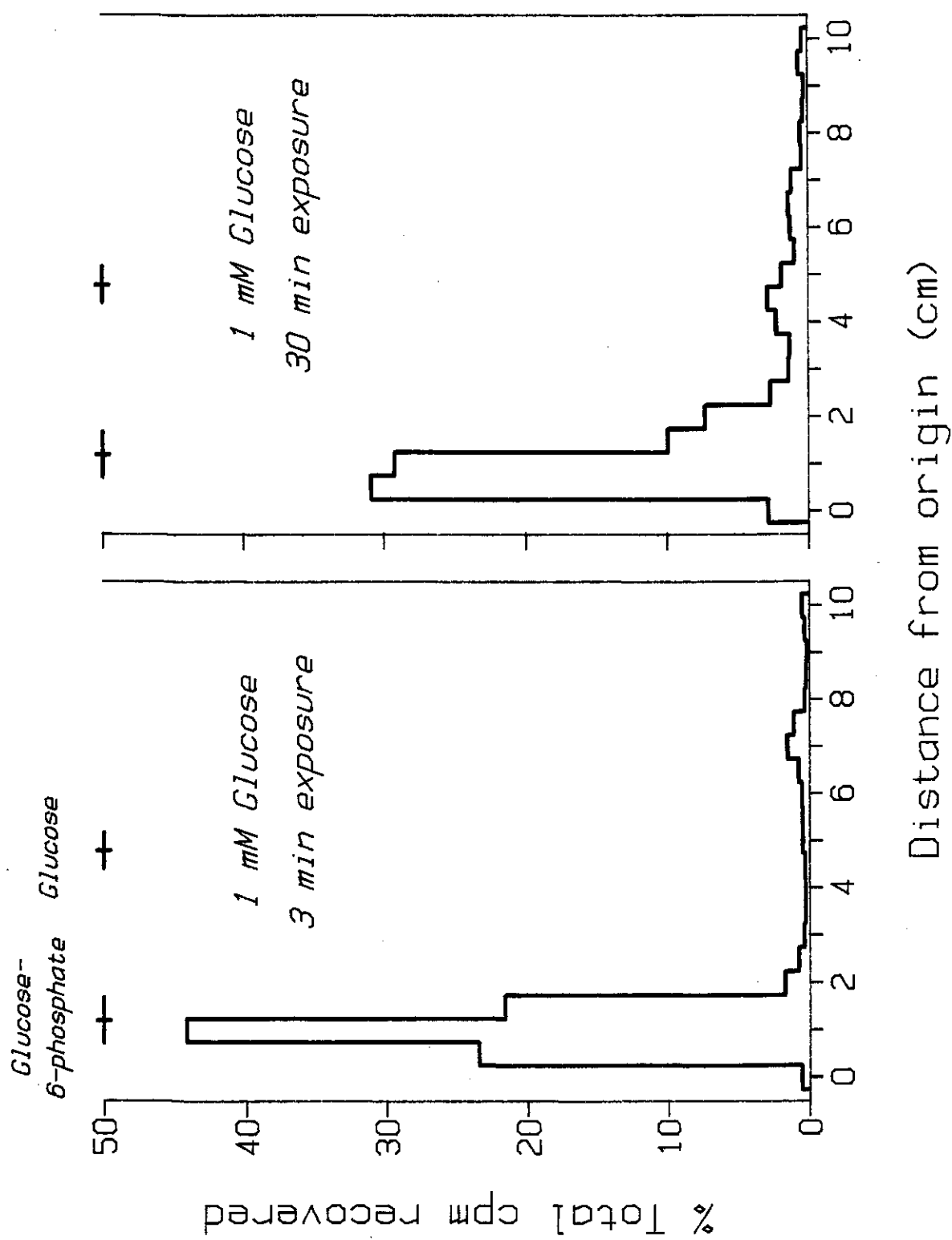
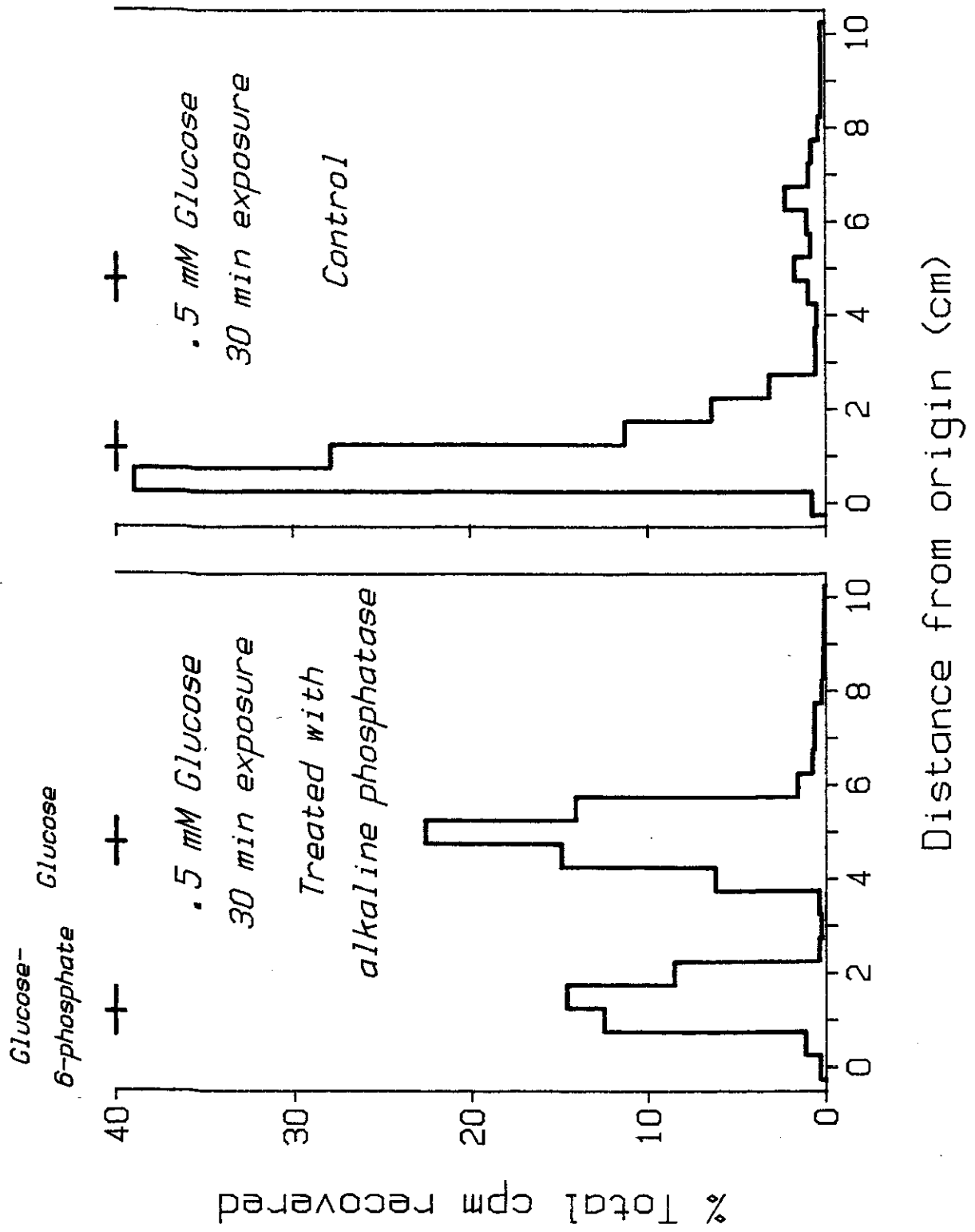


FIG. IV-8c. Radiochromatogram of ethanol extracts of midgut of Callinectes sapidus from mucosal glucose uptake experiments using 0.5 mM glucose: effect of alkaline phosphatase. The ethanol extracts were pre-treated with alkaline phosphatase or the heat-treated enzyme (control). See legend of Fig. IV-8a for further explanations.



DISCUSSION

The present study demonstrates that glucose enters the mucosal border of the midgut of Callinectes sapidus via two routes: (1) a Na^+ -dependent phlorizin-sensitive mechanism, and (2) a Na^+ -independent non-saturable pathway that probably represents diffusion into the cells. The inhibitory effects of metabolic inhibitors and ouabain on mucosal uptake are the same as for transmural fluxes (Chapter III). Thus, these findings strengthen the former suggestion that the regulation of intracellular Na^+ by $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ is necessary for mucosal entry of glucose.

The net transmural glucose flux across the midgut in a glucose concentration of 5 mM is about $40 \text{ nmol cm}^{-2} \text{ h}^{-1}$ (Chapter III). The total mucosal glucose uptake in the same substrate concentration, as calculated from Equation (2), is $60 \text{ nmol cm}^{-2} \text{ h}^{-1}$. Thus, more than 50% of the glucose entering the mucosal border of the midgut epithelium is subsequently translocated to the serosal medium. Most of this net glucose delivery is in the form of free glucose (Chapter III). However, the present study shows that most glucose accumulated by the midgut is in the form of phosphorylated compound(s), probably representing various stages of metabolic degradation. The same observation has been made in the midgut of the freshwater shrimp, Macrobrachium rosenbergii (Ahearn & Maginniss, 1977). This phenomenon is in contrast to glucose transport in the mammalian intestine. In the latter system, free glucose is accumulated to a high level inside the epithelium so that the serosal

exit of glucose can simply be explained by a diffusive pathway (see Crane, 1968 for a review). Since experimental studies indicate that the intracellular glucose concentration is low in the crustacean midgut, an alternative mechanism to account for the translocation of glucose in the serosal border is necessary.

Ahearn & Maginniss (1977) proposed two hypotheses for the transmural transport of glucose through the midgut of M. rosenbergii. The first model suggests that the two steps of the glucose transport process in the luminal and basolateral membranes are linked to phosphorylation and dephosphorylation, respectively. The phosphorylation-dephosphorylation hypothesis originally proposed to account for active glucose absorption in mammalian intestine was abandoned as a result of more recent evidence (see Crane, 1960 for a review). Phosphorylation-linked sugar transport, however, has been demonstrated in bacteria (Simoni et al., 1967; Kaback, 1968) and yeast (van Steveninck, 1968, 1969, 1970, 1972; Jaspers & van Steveninck, 1975). The present finding of the accumulation of phosphorylated compounds in crustacean midgut cannot be taken as conclusive evidence for this hypothesis since many cells increase the concentration of metabolic intermediates on the supply of nutrients.

The second model proposes that part of the free glucose that passes through the luminal membrane is rapidly metabolized and the rest is actively translocated across the basolateral membrane via a high-affinity glucose carrier. The presence of a serosal sugar pump has been suggested in the rat intestine (Esposito, Faelli & Capraro, 1973). The results of

the present study are compatible with both proposed models and do not favor either. Studies on glucose fluxes at the serosal border are necessary to clarify the glucose efflux mechanism.

The kinetic constants, K_m and J_{max} , of the Na^+ -dependent glucose uptake by the midgut of Callinectes sapidus are in the same range as those determined in the same tissue of M. rosenbergii (Ahearn & Maginiss, 1970). High-affinity transport systems with low maximal transport rates for amino acids are also present in the midgut of the same species (Brick & Ahearn, 1978), and the marine shrimp, Penaeus marginatus (Ahearn, 1974, 1976). Ahearn (1982) pointed out that the half-saturation constants of nutrient transport mechanisms in the crustacean midgut are one or two orders of magnitude lower than those in the mammalian intestine. In addition, the maximal transport rates in the midgut are markedly lower. He suggested that the low J_{max} limits the nutrient absorptive role of the midgut and the high-affinity nature is adaptive to the presumably low nutrient levels in the midgut lumen, resulting from predominant nutrient absorption by the hepatopancreas. The findings of the present study support this suggestion. With a low luminal glucose concentration, nutrients taken up by the midgut may be largely utilized for generating energy to support other physiological functions that have been suggested for this tissue, such that net transmural nutrient absorption is minimal. These proposed functions include secretion of the peritrophic membrane (Forster, 1953; Georgi, 1969; Johnson, 1980), and fluid absorption during ecdysis (Mykles & Ahearn, 1978; Mykles, 1980).

The hypothesis that the midgut does not play a significant role in total nutrient absorption rests upon the comparison of kinetic studies on the gut of different animal species. It should be emphasized that the kinetic constants determined in these studies represent estimates made under different experimental conditions, and thus are subjected to variations. For example, kinetic constants of glucose uptake by mammalian intestine are dependent on luminal sodium ion concentration (Crane, Forstner & Eichholz, 1965; Bihler, 1969; Hopfer, 1977). Further, the extent of the unstirred layer, which tends to vary in different in vitro preparations, also affects the values estimated for the half-saturation constant (Wilson & Dietschy, 1974; Thomson & Dietschy, 1980). Therefore, the differences in experimental techniques adopted make the comparison of kinetic constants difficult. Accordingly, hypotheses based on comparison of kinetic data from different laboratories remain speculative. Recently, there have been kinetic studies on gut transport processes of different species under identical experimental conditions (Thomson, Hotke & Weinstein, 1982; Ferrais & Ahearn, 1983; Karasov, Solberg & Diamond, 1983). This relatively new approach to comparative gastrointestinal physiology would be rewarding in elucidating the importance of the crustacean midgut in nutrient absorption.

CHAPTER V

GENERAL CONCLUSIONS

In this study, I have described some characteristics of sodium and glucose transport in the gut of Callinectes sapidus. The major original findings are summarized as follows:

1. Both midgut and hindgut of C. sapidus exhibit a low serosa-positive potential difference.
2. There is a net sodium absorption across the midgut. Since salinity acclimation does not change the rate of absorption, its role in sodium hyperegulation is minimal. A decrease of passive sodium permeability, however, is likely to limit sodium loss through the midgut in low salinity waters.
3. Probably due to the presence of a chitinous lining, sodium exchange across the hindgut is small. Thus, its importance in sodium balance is negligible.
4. The midgut is capable of net transport of free glucose from lumen to hemolymph. This process is saturable, energy- and sodium-dependent, and phlorizin-sensitive. These characteristics are similar to those exhibited by the mammalian intestine.
5. The mucosal glucose uptake by the midgut comprises two components: (1) a saturable component that is sodium-dependent, and (2) a non-saturable component that is believed to represent passive flows into the cells.
6. Glucose taken up by the midgut is rapidly metabolized as phosphorylated forms. Thus the serosal efflux of free glucose probably requires an active process.

7. The kinetic constants of mucosal glucose uptake by the midgut limit its glucose absorbing capability. This limitation suggests that nutrient uptake by the midgut does not contribute a significant portion of total nutrient absorption. A salvage function of the midgut in nutrient uptake is probable.

This study showed that the role of the midgut of Callinectes sapidus in osmoregulation and nutrient absorption is small. Thus, the function of this tissue is still largely unknown. Other functions that have been suggested for the crustacean midgut are secretion of the peritrophic membrane (Forster, 1953; Georgi, 1969), and fluid absorption during ecdysis (Mykles & Ahearn, 1978; Mykles, 1980). Johnson (1980) also ascribed peritrophic membrane formation to be a function of the midgut epithelium of C. sapidus. In Homarus americanus, net fluid absorption through the midgut is sodium-dependent (Mykles, 1981). In Macrobrachium rosenbergii, this uptake is dependent on the presence of both sodium and chloride (Ahearn et al., 1977). Further, variation of net sodium flux across the midgut during the molt cycle correlates well with that of net fluid absorption (Kullama, 1981). The present study demonstrated a net sodium flux in the midgut of C. sapidus. The role of this route of sodium entry in fluid transport as well as changes of these transport processes during the molt cycle of C. sapidus remain to be elucidated.

APPENDIX A

VARIATIONS OF HEMOLYMPH OSMOTIC PRESSURE AND IONIC CONCENTRATIONS
IN CALLINECTES SAPIDUS WITH ACCLIMATED SALINITY

INTRODUCTION

The common blue crab, Callinectes sapidus Rathbun is distributed along the Atlantic coast of North and South America, from Nova Scotia to northern Argentina (Williams, 1974). The centers of distribution are in the estuarine systems along the east coast of the United States. With a wide range of salinity tolerance, the species is known to occur in habitats ranging from freshwater to hypersaline lakes (Gunter, 1938; Odum, 1953; Gifford, 1962; Mangum & Amende, 1972). In addition, the life history of the animal involves migration between waters of different salinities (Churchill, 1920; Van Engel, 1958; Darnell, 1959; Tagatz, 1968; Dudley & Judy, 1973). Its osmoregulatory ability in these habitats is well documented. Like many estuarine crustaceans, it is roughly isosmotic in seawater and regulates hyperosmotically at dilute salinities (Tan & Van Engel, 1966; Ballard & Abbott, 1969; Tagatz, 1971; Gerald & Gilles, 1972; Lynch, Webb & Van Engel, 1973; Findley & Stickle, 1978). Variations of hemolymph ionic composition with external salinity have also been studied (Odum, 1953; Mantel, 1967; Colvocoresses, Lynch & Webb, 1974; Gerald & Gilles, 1972; Findley & Stickle, 1978). This section of

my dissertation confirms the reported studies by determining osmotic pressure and ionic concentrations in the hemolymph of blue crabs acclimated in waters of different salinities. These data form the basis for the composition of physiological salines used in the in vitro perfusion experiments.

MATERIALS AND METHODS

Acclimation of animals

The blue crabs were purchased from Gulf Specimen Co., Inc., Panacea, Florida and air-shipped to Woods Hole, Massachusetts. They were maintained inside individual cages in running seawater at $20 \pm 1^{\circ}\text{C}$ for 7 d before acclimation. Each animal was fed daily with 4-5 freeze-dried krill. All animals used in this study were at intermolt, with a carapace width of greater than 10 cm.

The animals were acclimated in waters of different salinities in 100 l insulated tanks. Media of lower salinity than seawater were prepared by mixing full-strength seawater with fresh water. Media of higher salinity than seawater were prepared by adding Instant Ocean to seawater. Inside the tanks, media were aerated and maintained at $20 \pm 1^{\circ}\text{C}$ with aquarium heaters. Wastes were removed with charcoal filters. No more than four crabs were acclimated in a tank at a time. The animals were acclimated in a stepwise fashion for 2 d until the desired salinity was attained. Preliminary experiments showed that at 7 o/oo S, the

lowest salinity used in this study, hemolymph osmotic pressure was stabilized after 7 d of acclimation. Thus, animals at all salinities were acclimated for 7 to 14 d before sampling of hemolymph.

Collection of serum samples

Hemolymph samples were obtained by cutting through the meropodite of the right fourth pereopod and collecting the dripping hemolymph in a test tube. The clot formed was broken up with a glass rod. The sample was then centrifuged for 20 min in a clinical centrifuge. The supernatant serum was pipetted to a capped polyethylene vial. Sample volumes varied from 1 to 3 ml. Samples of the acclimated media were taken concurrently with hemolymph samples. All samples were frozen until analysis.

Methods of analysis

Osmotic pressure of serum and seawater samples was determined with a Wescor vapor pressure osmometer. Levels of sodium, potassium, calcium, and magnesium were determined with a Jarrel-Ash atomic absorption spectrophotometer. Chloride concentration was determined with a Buchler-Cotlove chlorimeter. Sulfate concentration was determined with a turbidimetric method (American Public Health Association, 1975), adapted for microsamples (Malley, 1977a).

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RESULTS AND DISCUSSION

Osmotic and ionic concentrations of the serum of Callinectes sapidus were plotted against those of the acclimated medium in Fig. A-1 through 7. A scale of the corresponding salinity (± 2 o/oo S) is also illustrated in each figure.

Serum osmotic pressure was maintained at the same level as the medium above about 25 o/oo S (Fig. A-1). Below this point, serum osmotic pressure was well regulated above the medium although it had a tendency to decrease with salinity. At 7 o/oo S, the lowest salinity tested, the crabs had a serum osmotic pressure of about 70 % of those acclimated at 34 o/oo S. These findings are consistent with results of reported studies on animals in laboratory experiments (Ballard & Abbott, 1969; Gerald & Gilles, 1972; Findley & Stickle, 1978) and field studies (Mangum & Amende, 1972; Lynch et al., 1973).

Variation of serum sodium concentration closely paralleled that of serum osmotic pressure (Fig. A-2), except that serum sodium levels were slightly hyperionic in salinities where the serum was isosmotic to the medium. Mantel (1967) and Findley & Stickle (1978) also demonstrated hemolymph sodium concentration slightly above the medium at high acclimated salinities. Yet, Gerald & Gilles (1972) showed essentially isoionic regulation in this range. Chloride regulation in low salinities also paralleled change in total osmotic pressure (Fig. A-3). Above roughly 30 o/oo S, however, the serum chloride concentration was lower than that of the medium. Such a pattern of chloride regulation has

FIG. A-1. Relation between the osmotic pressure of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 7-10 samples, and bars represent \pm SEM. No bars shown means that they are within the dots. The lower axis indicates the approximate corresponding salinity (± 2 o/oo S).

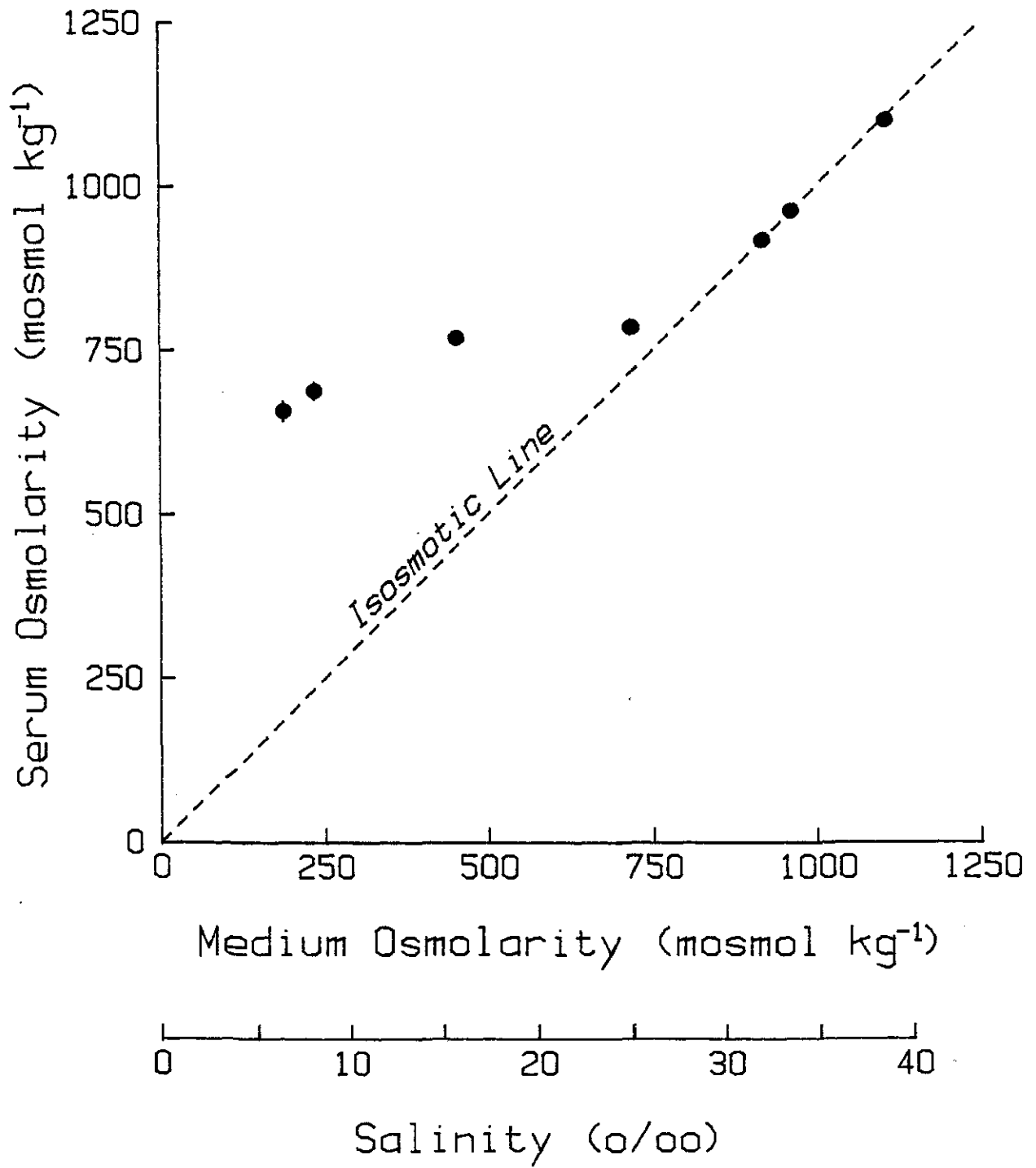


FIG. A-2. Relation between the sodium concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 7-10 samples, and bars represent \pm SEM. For further explanations, see legend of Fig. A-1.

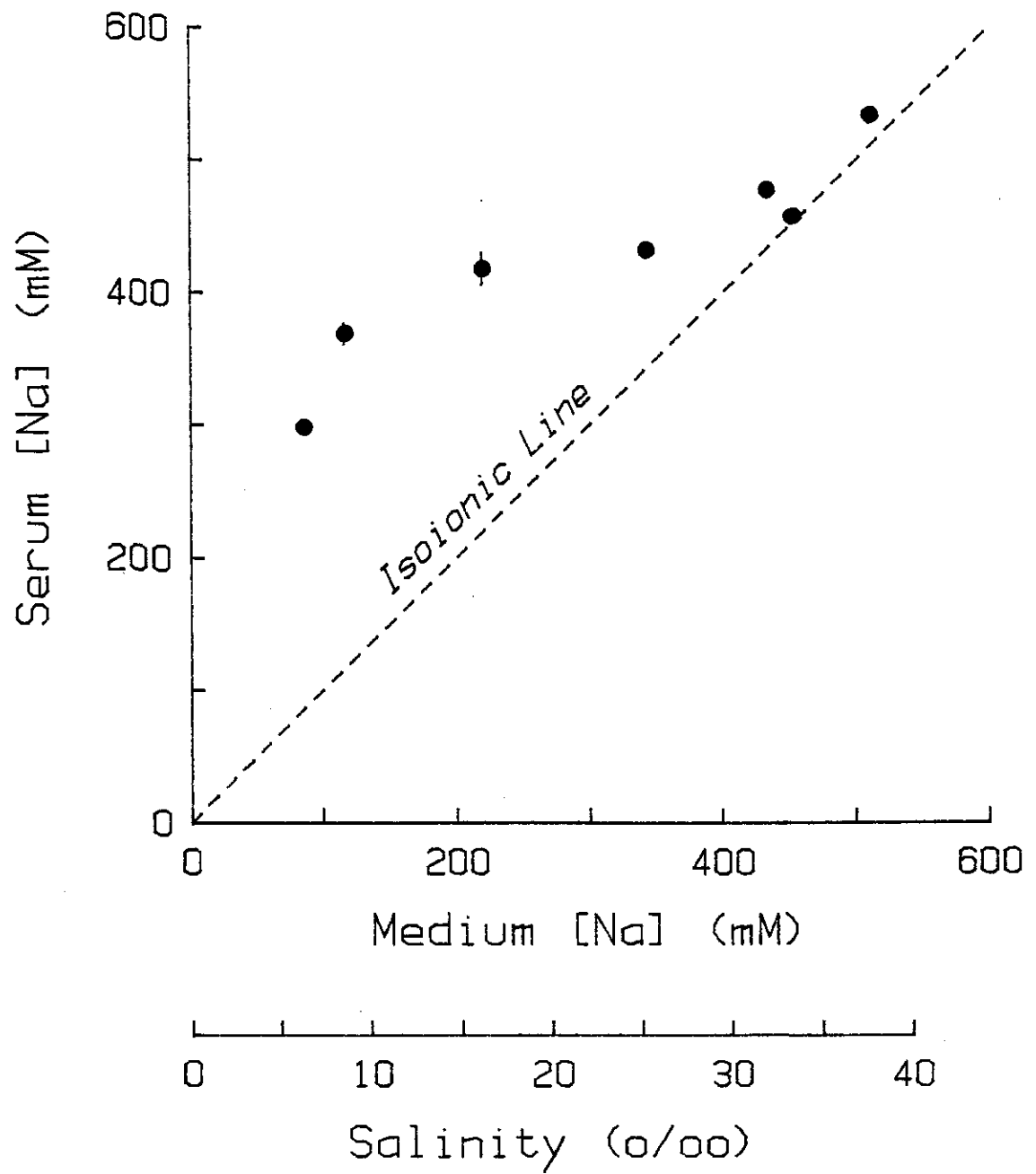
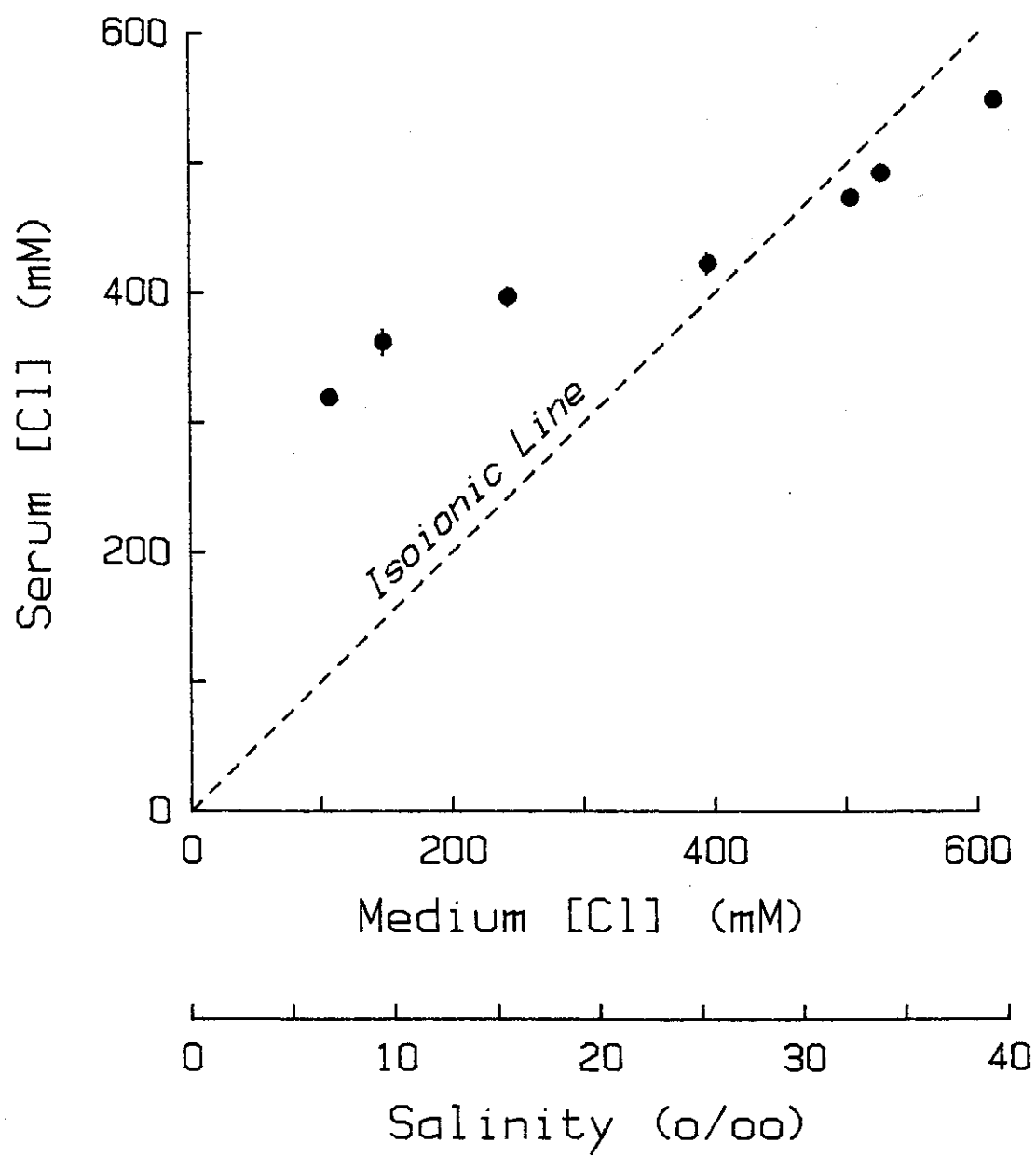


FIG. A-3. Relation between the chloride concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 6-10 samples, and bars represent \pm SEM. For further explanations, see legend of Fig. A-1.



been observed previously (Odum, 1953; Gerald & Gilles, 1972; Findley & Stickle, 1978). Sodium and chloride concentrations determined in the present study are within the range of published data on animals collected in the field without prior acclimation (Gifford, 1962; Lynch et al., 1973; Colvocoresses et al., 1974).

Serum potassium was more concentrated than the ambient seawater over the range of salinities studied (Fig. A-4). There was a general trend of decreased serum potassium with reduced salinity. These results are in accord with Gerald & Gilles (1973), although the medium potassium concentration that they used never exceeded 10 mM. In high salinity waters, Gifford (1962), Colvocoresses et al. (1974) and Findley & Stickle (1978) found lower potassium concentration in the hemolymph than the medium.

Serum calcium was hyperionic and magnesium hypoionic to the medium in all salinities studied (Fig. A-5 and 6). The levels of these two cations are within the range reported in field study by Colvocoresses et al. (1974), who also showed hyperionic magnesium regulation below 6-8 o/oo S, which is beyond the range of the present study. It is noted that serum magnesium concentration in C. sapidus was usually less than 50% of that in the medium. Such low serum magnesium levels are often associated with more active crustaceans (Robertson, 1960b; Lockwood, 1972). Robertson (1960b) ascribed regulation of a low hemolymph magnesium concentration to its anesthetic effect on the neuromuscular system.

Serum sulfate concentrations were hypoionic to the medium and they tended to decrease linearly with salinity (Fig. A-7). Sulfate regulation in C. sapidus has not been previously studied.

FIG. A-4. Relation between the potassium concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 6-11 samples, and bars represent \pm SEM. For further explanations, see legend of Fig. A-1.

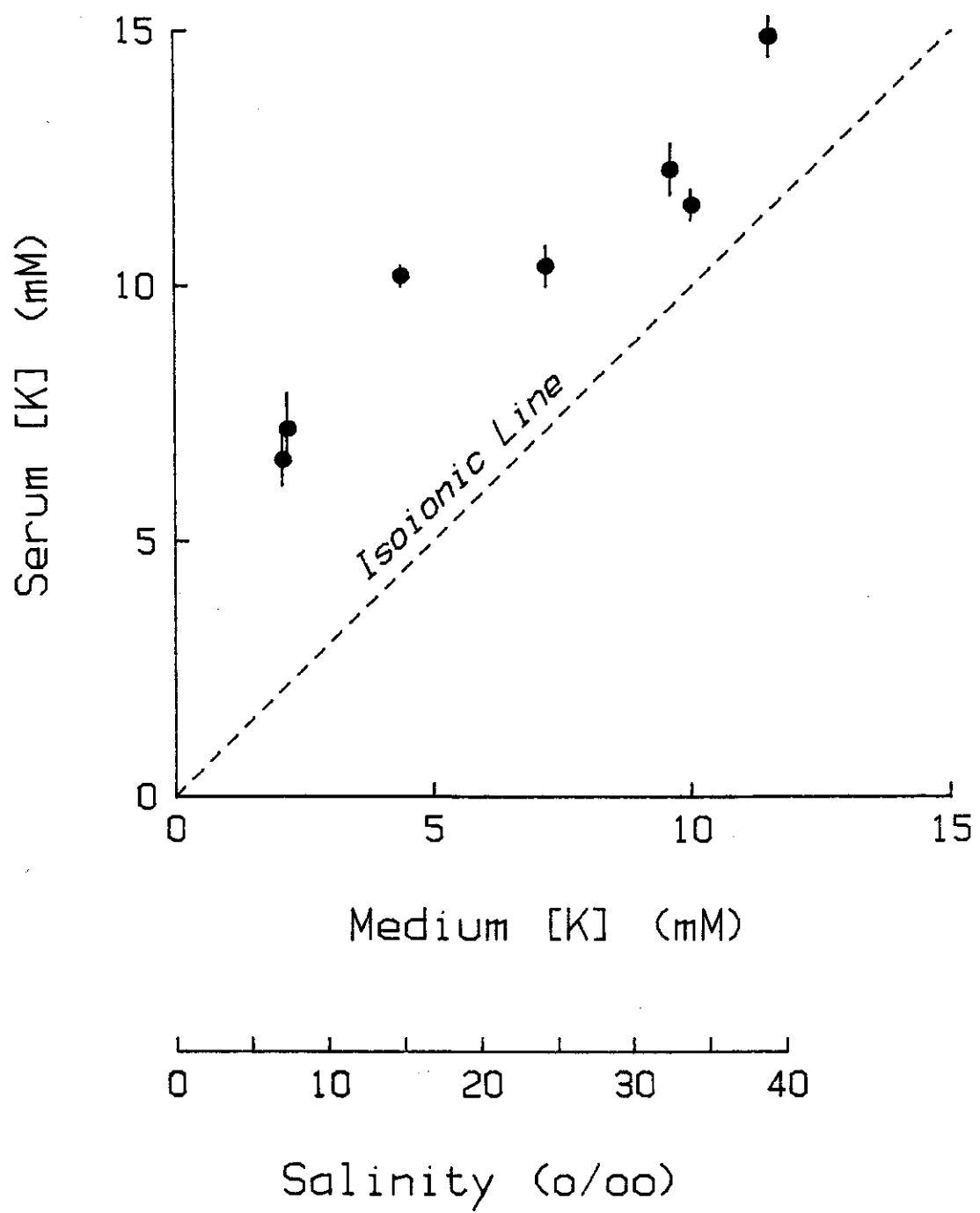


FIG. A-5. Relation between the calcium concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 9-13 samples, and bars represent \pm SEM. For further explanations, see legend of Fig. A-1.

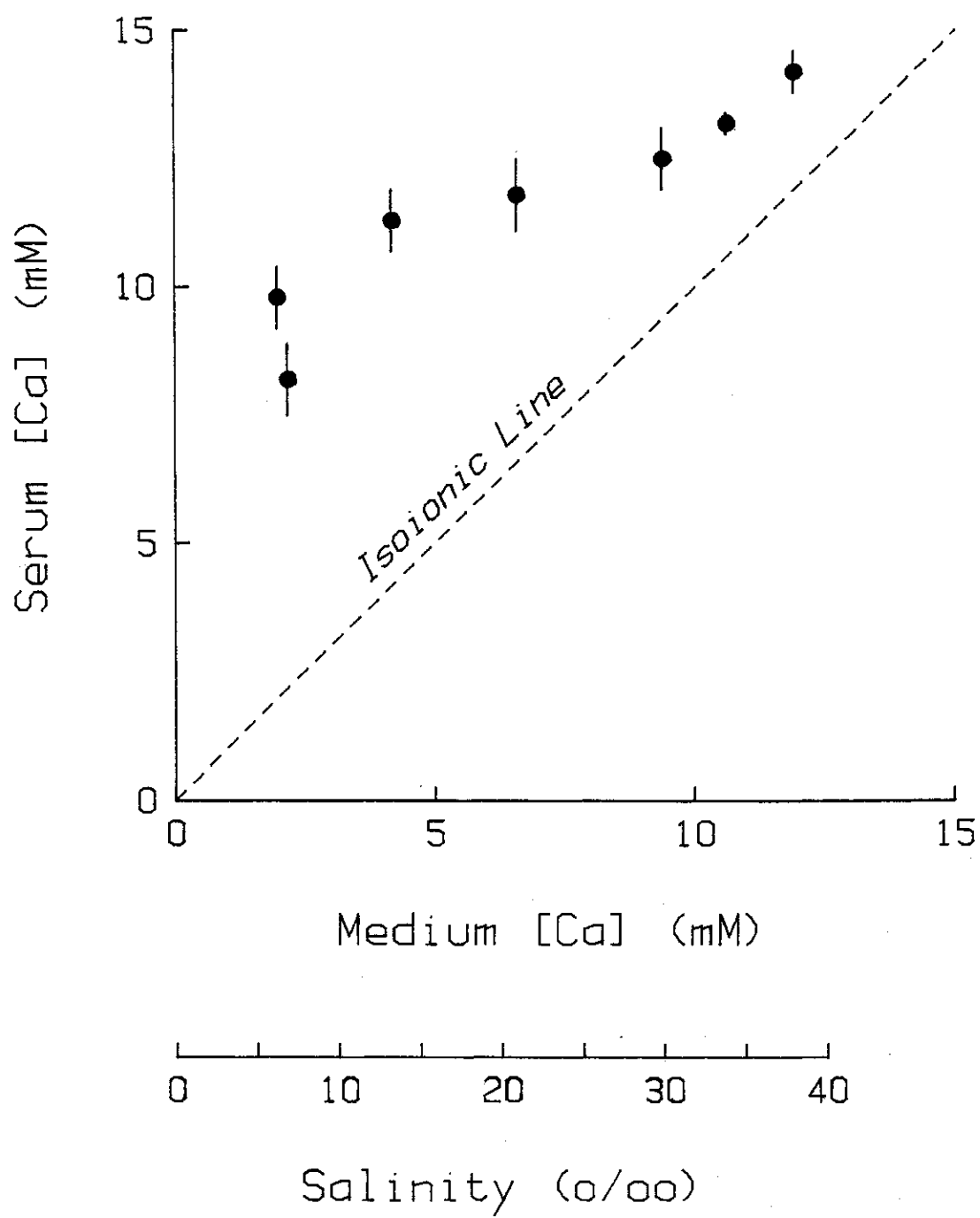


FIG. A-6. Relation between the magnesium concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 6-13 samples, and bars represent \pm SEM. For further explanations, see legend of Fig. A-1.

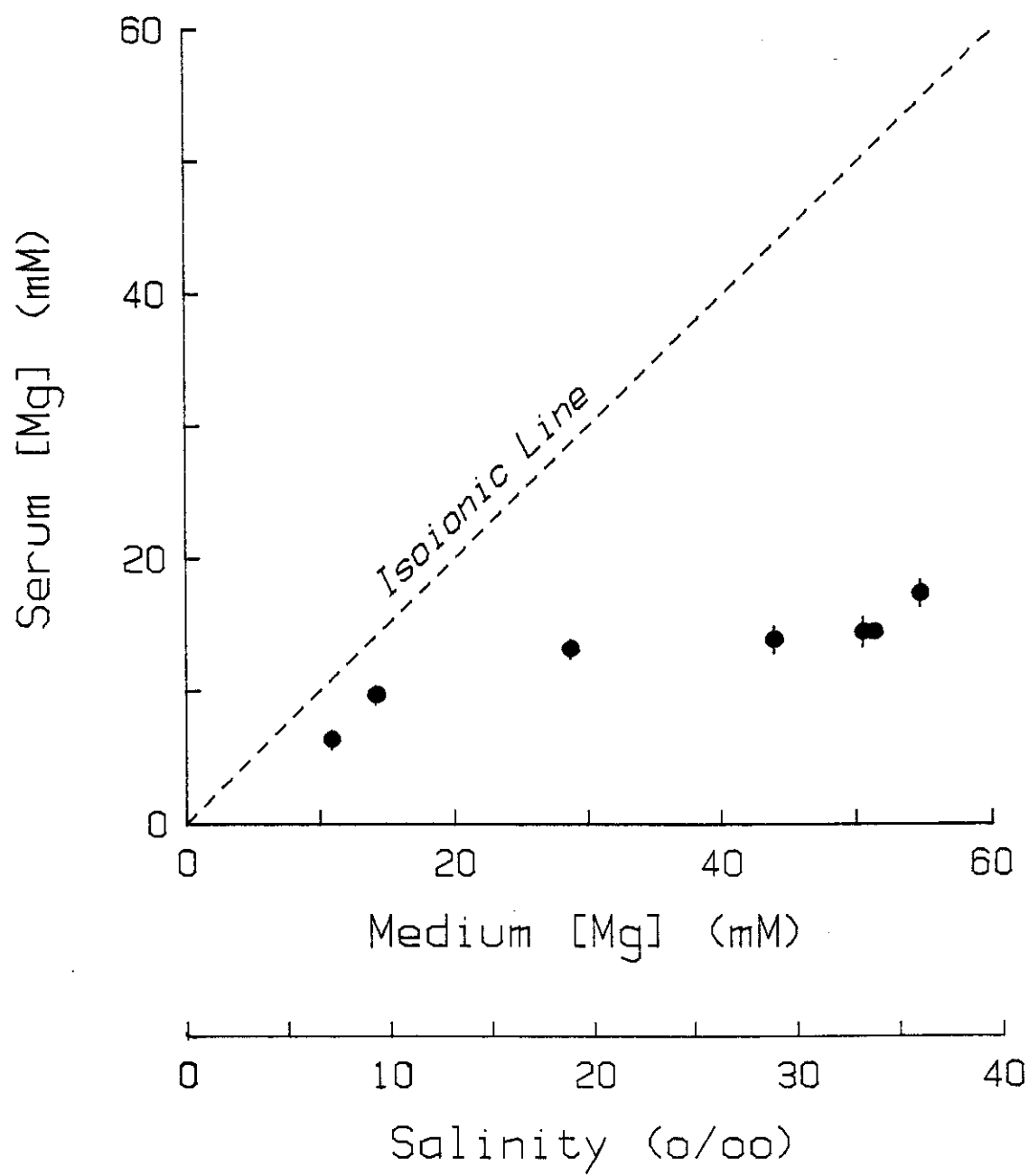
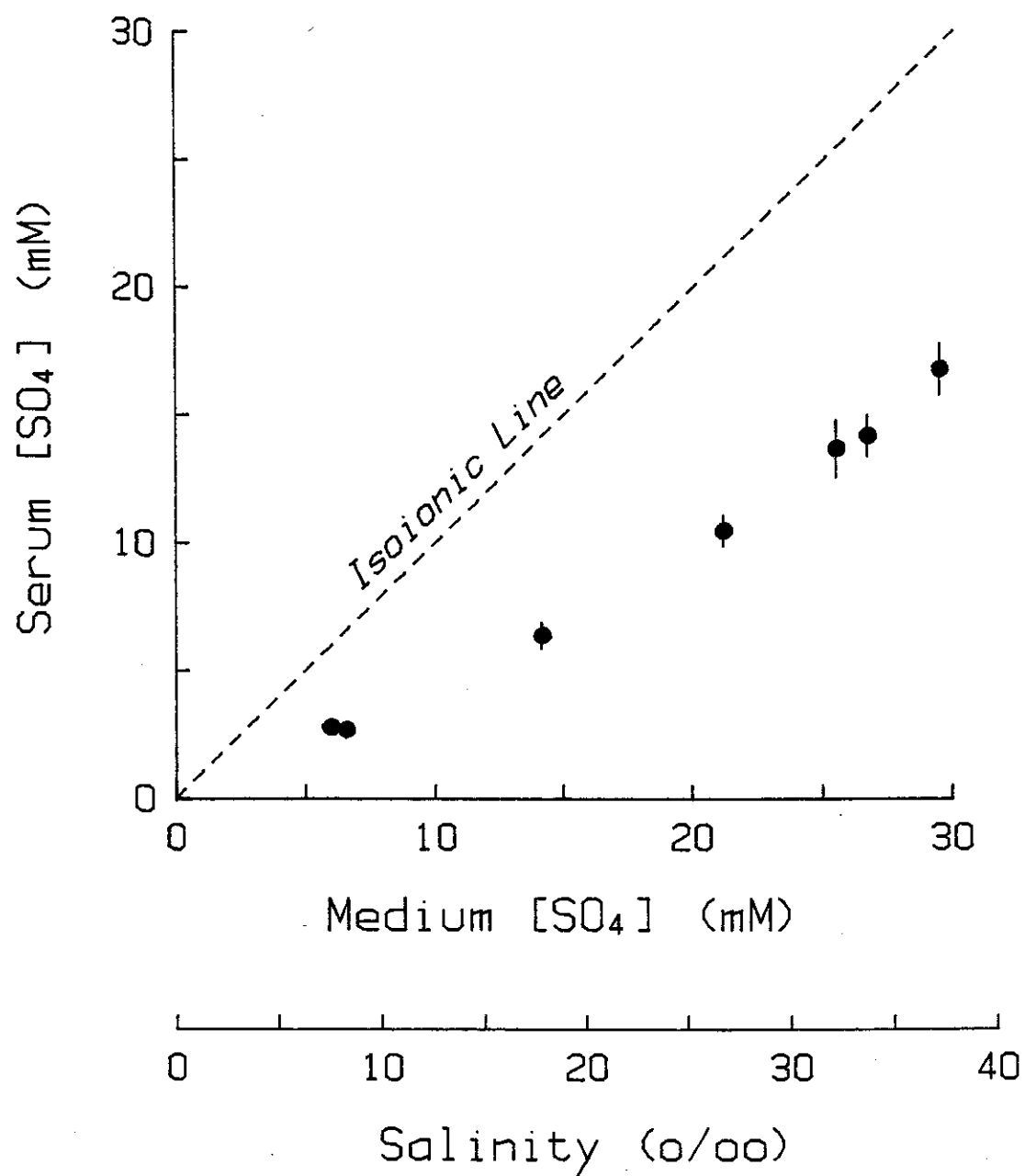


FIG. A-7. Relation between the sulfate concentration of serum in Callinectes sapidus and the ambient medium. Data points are the mean of 5 or 10 samples, and bars represent \pm SEM. For further explanations, see legend of fig. A-1.



In conclusion, the osmotic pressure and ion concentrations reported in the present study are generally consistent with the literature. There are some disagreements among different studies, especially in extreme salinities. These discrepancies may be due to various factors including differences in analytical techniques, temperature and length of time of acclimation (Engels et al., 1974), seasonal variations which may or may not be temperature-related (Lynch et al., 1973; Colvocoresses et al., 1974), molt stage of the animals studied (Tagatz, 1971), and intrinsic differences in hemolymph composition among populations in different localities.

The compositions of serum, ambient seawater, and physiological saline used in in vitro perfusion experiments of C. *sapidus* acclimated in 34 o/oo and 7 o/oo S are shown in Table A-1 and 2, respectively.

TABLE A-1

Compositions of serum, ambient seawater, and physiological saline used in experiments of Callinectes sapidus acclimated in 34 o/oo S

Constituent	Serum	Seawater	Physiological saline
Na	457.3 \pm 2.7	451.6	460
K	11.6 \pm 0.3	10.0	11
Ca	13.2 \pm 0.2	10.6	13
Mg	14.5 \pm 0.4	51.3	13
Cl	492.6 \pm 6.0	525.6	497
SO ₄	14.1 \pm 0.8	26.7	13
HEPES	-----	----	5
Mannitol	-----	----	50
Osmotic pressure	965 \pm 4	963	960

Values are expressed in mM except for osmotic pressure in mosmol kg⁻¹. Values for serum represent mean \pm SEM of ten animals.

TABLE A-2

Compositions of serum, ambient seawater, and physiological saline used in experiments of Callinectes sapidus acclimated in 7 o/oo S

Constituent	Serum	Seawater	Physiological saline
Na	298.4 \pm 4.0	86.5	300
K	6.6 \pm 0.5	2.1	7
Ca	8.2 \pm 0.7	2.2	8
Mg	6.3 \pm 0.7	10.9	6
Cl	319.0 \pm 5.0	107.0	329
SO ₄	2.8 \pm 0.2	6.0	3
HEPES	-----	----	5
Mannitol	-----	----	50
Osmotic pressure	659 \pm 15	189	660

Values are expressed in mM except for osmotic pressure in mosmol kg⁻¹. Values for serum represent mean \pm SEM of ten animals.

APPENDIX B

OXYGEN CONSUMPTION RATES OF ISOLATED
MIDGUT AND HINDGUT OF CALLINECTES SAPIDUS

To demonstrate the viability of the isolated gut tissues in the in vitro perfused experiments, the oxygen consumption rates (Q_{O_2}) of midgut and hindgut excised from the blue crabs were determined with a Gilson differential respirometer. In each experiment, the isolated midgut and hindgut from 3 or 4 animals were each placed into reaction flasks with 5 ml of physiological saline containing 5 mM glucose. The preparations were allowed to incubate in the respirometer for 30 min before measurements started. All measurements were made at 20°C. Manometer readings were taken at 30 min intervals. After 60 min, the reaction flasks were removed from the respirometer. To some tissues, 0.1 ml of 50 mM potassium cyanide stock solution was added to achieve a final concentration of 1 mM. The same volume of 50 mM potassium chloride stock solution was added to other tissues acting as controls. The reaction flasks were placed back to the respirometer and equilibrated for about 25 min before Q_{O_2} were measured for another hour. At the end of the experiment, the tissues were removed from the flasks, blotted, and the wet weights were determined. Values of Q_{O_2} reported were expressed as mean \pm SEM (no. of tissues).

During the first hour of the experiment, the Q_{O_2} of midgut and hindgut were 1.0 ± 0.2 (10) and 0.38 ± 0.06 (10) $\mu\text{l O}_2 \text{ mg wet wt.}^{-1} \text{ h}^{-1}$, respectively. The midgut has a significantly higher rate than the hindgut ($P < .01$). These rates compare favorably with the reported value of $0.59 \mu\text{l O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ for the midgut of Penaeus marginatus (Ahearn, 1974). Upon addition of cyanide, Q_{O_2} of Callinectes midgut dropped rapidly to zero (Fig. B-1). Control tissues maintained a stable rate throughout the experimental period. Q_{O_2} decrease of the hindgut in response to cyanide was less evident (Fig. B-2), but the rate was significantly lower than the control at the end of the experiment ($P < .01$).

These experiments showed that the isolated midgut and hindgut maintained a stable oxygen consumption rate for more than 2 h after the tissues were excised from the animals. Thus, they provided evidence that the tissues did not deteriorate significantly during the in vitro perfusion experiments.

FIG. B-1. Time course of oxygen consumption rate of the isolated midgut of Callinectes sapidus. Each point is the mean of 5 measurements, and the bar represents \pm SEM. In some cases, half of the bar is omitted for clarity. Asterisks indicate significant differences between the rate exhibited by tissues treated with cyanide and that by the controls.

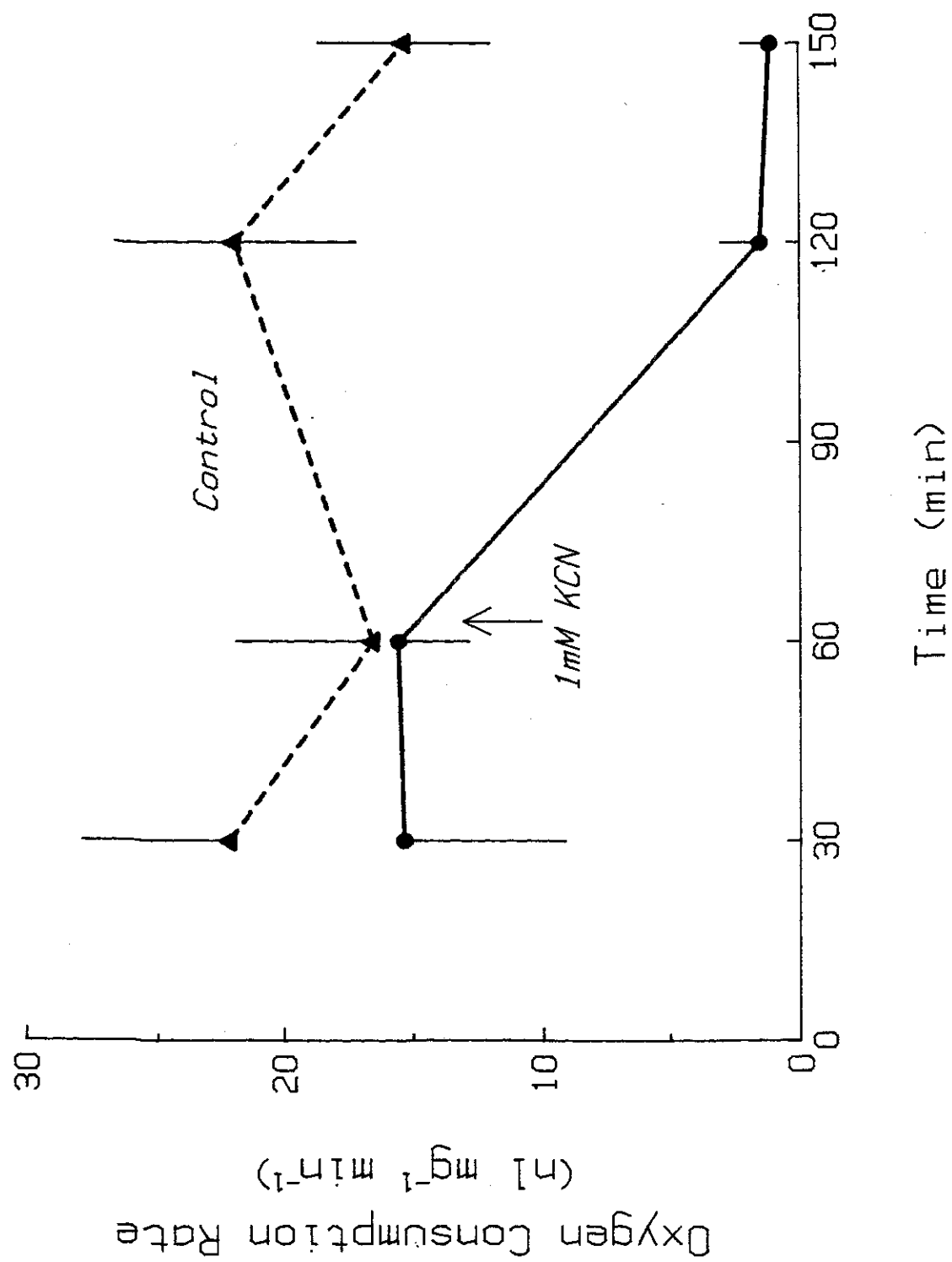
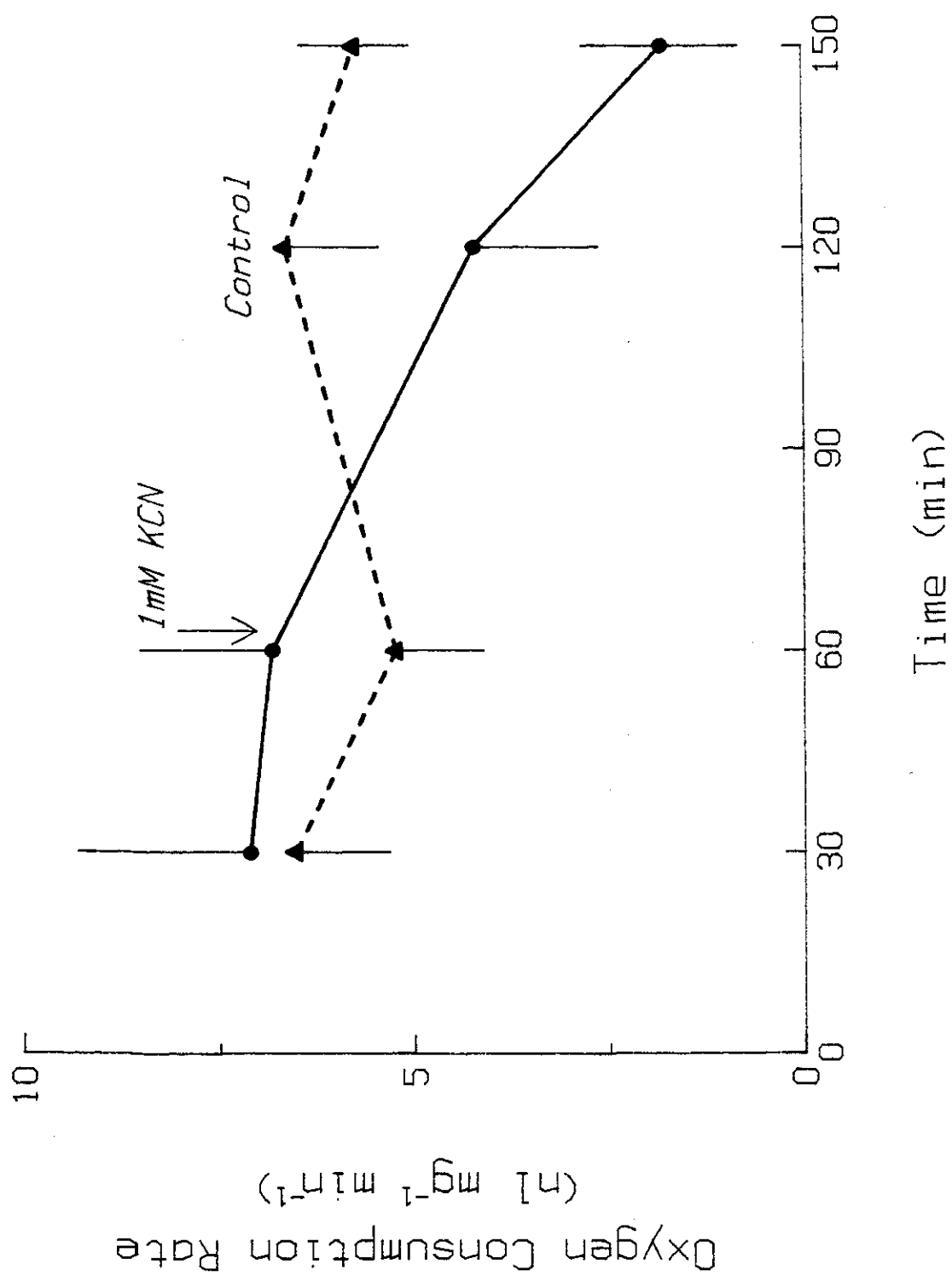


FIG. B-2. Time course of oxygen consumption rate of the isolated hindgut of Callinectes sapidus. See legend of Fig. A-8 for explanations.



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